Mitochondrial function in the evolutionary origin of the female germ line

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

August 2013

Statement of originality

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Abstract

Oxidative phosphorylation couples ATP synthesis to respiratory electron transport. This coupling occurs in mitochondria, which carry DNA. Respiratory electron transport in the presence of molecular oxygen generates mutagenic reactive oxygen species (ROS) at a frequency that is itself increased by mutation. Damage to mitochondrial DNA (mtDNA) therefore accumulates within the lifespan of individual organisms. Syngamy requires motility of one gamete, and this motility requires ATP. It has been proposed that that oxidative phosphorylation is absent in the special case of quiescent, template mitochondria, and that these remain sequestered in oocytes and female germ lines. Oocyte mtDNA is thus protected from damage. Here I present evidence that female gametes, which are immotile, repress mitochondrial DNA transcription, mitochondrial membrane potential ($\Delta\Psi_m$), and ROS production. In contrast, somatic cells and male gametes are seen actively to transcribe mitochondrial genes for respiratory electron carriers, and to produce ROS. I find that this functional division of labour between sperm and egg is widely distributed within the animal kingdom, and characterised by contrasting mitochondrial size and morphology. If quiescent oocyte mitochondria alone retain the capacity for an indefinite number of accurate replications of mtDNA, then "female" can be defined as that sex which transmits genetic template mitochondria. Template mitochondria then give rise to mitochondria that perform oxidative phosphorylation in somatic cells and in male gametes of each new generation. Template mitochondria also persist within the female germ line, to populate the oocytes of daughters. Thus mitochondria are maternally inherited.

This thesis is dedicated to the memory of
my father Dr. Wilson Marcelino de Paula

Acknowledgements

I would like to express my heartfelt gratitude to my supervisor Prof. John Allen, who is not only my mentor but also a dear friend, and who welcomed me with open arms into his research laboratory. I would also like to extend my gratitude to my co-supervisor Dr. Brendan Curran, and Mrs. Carol Allen for their immense support, kindness and patience throughout my PhD research.

I am thankful to my dear lab mates and friends Dr. Iskander Ibrahim, Dr. Sujith Puthiyaveetil, Mr. Ahmed-Noor Agip and Mrs. Liang Wang for the joyful moments, insightful scientific discussions, and especially for their strong companionship during difficult times.

I sincerely thank Prof. Bill Martin, Dr. Nick Lane, Dr. Janneke Balk and Dr. Markus Schwarzländer for their kind advice and enlightening scientific discussions during these years. I also thank Dr. Fanis Missirlis, Dr. Cathy Lucas, Dr. Rachel Ashworth and Dr. Gema Vizcay-Barrena for their invaluable assistance to my experimental work.

I want to express my eternal gratitude to my loving family, especially to my mother Magaly, who made sure I was raised as a freethinker in a place where it does not happen very often. My love and thanks to my vovó Odelza, tia Fernanda, and Flávia. I am also very grateful to Garik, Marina, Janna, Diana and Hrach for their continuous support and encouragement.

Finally, I want to thank my beautiful wife Marianna, for her unconditional love and inexhaustible understanding throughout my entire PhD research.

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Abbreviations

$^{\cdot}OH$	hydroxyl radical ion
$\Delta\Psi_m$	mitochondrial proton membrane potential
ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
ArcB	anoxic redox control protein B
ATP	adenosine 5'-triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
CKS	cyclin-dependent kinase subunit
cob	cytochrome reductase subunit b
CoRR	co-location for redox regulation
cox1	cytochrome oxidase subunit 1
CSK	chloroplast sensor kinase
CuZn-SOD	copper/zinc superoxide dismutase
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamidine
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
ecSOD	extracellular superoxide dismutase
ETC	electron transport chain
Fe-S	iron-sulphur cluster
Fe-SOD	iron superoxide dismutase
FRS	fellow of the Royal Society
FRTA	free radical theory of ageing
GV	germinal vesicle
Gyr	giga years, or 10^9 years
H_2	molecular hydrogen
H_2DCF-DA	dichlorodihydrofluorescein diacetate
H_2O	water
H_2O_2	hydrogen peroxide

H_2S	hydrogen sulphide
IVF	*in vitro* fertilisation
kDa	kilo Dalton
kV	kilo Volt
LUCA	last universal common ancestor of cells
min	minutes
mM	millimolar
Mn-SOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MTA	mitochondrial theory of ageing
mtDNA	mitochondrial deoxyribonucleic acid
mtSSB	mitochondrial single-stranded DNA binding protein
mV	millivolt
nad1	NADH:ubiquinone oxidoreductase subunit 1
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometres
$O_2^{\bullet-}$	superoxide radical ion
O_2	molecular oxygen
O_2^{2-}	peroxide dianion
^oC	degrees Celsius
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PGC	primordial germ cell
Pi	inorganic phosphate
PPP	oxidative pentose phosphate pathway
$Q^{-\bullet}$	ubisemiquinone
qPCR	quatitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase PCR

RFU	relative fluorescence units
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
ROS	reactive oxygen species
rpL32	large ribosomal protein 32
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
SEM	standard error of the mean
SOD	superoxide dismutase
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TPP^+	tetraphenylphosphonium
tRNA	transfer ribonucleic acid
V(c,m)	ratio of crista volume to mitochondrion volume
YFP	yellow fluorescent protein
μl	microliters
μm	micrometre
μM	micromolar

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1

General Introduction

In 1883, the German botanist Andreas Schimper wrote (Schimper, 1883):

"*...if it is confirmed that the plastids are not formed de novo in the oocytes, then their relation to the hosting organism would roughly resemble a symbiosis. Maybe the green plants really have their origin in the unification of a colourless organism with one that is evenly stained with chlorophyll.*"

1.1. Origin of mitochondria

1.1.1. The endosymbiotic theory

In 1883, Andreas Schimper observed that organelles arise solely by division of pre-existing structures of the same type, and proposed an unprecedented hypothesis to explain that phenomenon. He hypothesized a symbiotic relationship between a 'colourless' host cell with other 'chlorophyll stained' cells – later to be called chloroplasts by the author himself. This was the first published idea of our contemporary concept of the endosymbiotic origin of chloroplasts and mitochondria (Martin and Müller, 2007; Martin, et al., 1992; Schimper, 1883; Schimper, 1885). Only later in 1905, a Russian scientist named Constantin Mereschkowsky published a more refined theory for an endosymbiosis origin of chloroplast. Mereschkowsky made more explicit assumptions, such as that chloroplasts, which he termed chromatophores (colour bearers), are autotrophic descendants from cyanobacteria (then known as unicellular algae), and that nuclei have originated from an invasion of a small "micrococcus" into a larger heterotrophic amoeba-like host cell (Martin and Kowallik, 1999; Mereschkowsky, 1905). Chloroplasts are coloured and thus conspicuous by light microscopy as intracellular organelles in plant and algal cells. Therefore the evidence of a cyanobacterium being the ancestor for chloroplasts in photosynthetic eukaryotes, such as land plants, was much more obvious. On the other hand, mitochondria, also termed "chondriosomes", were less well characterised and little was known of their function.

In 1890, Richard Altmann, a Prussian histologist, proposed that bioblasts were microorganisms inhabiting a host cell's cytoplasm, and that they were the ultimate units of life (Altmann, 1890; Wallin, 1923). Although Altmann's ideas may seem to be the earliest proposal for an endosymbiotic origin of mitochondria (Wallin, 1923), it is unlikely that bioblasts, as he described, were actual mitochondria or chondriosomes. Therefore priority for the earliest proposal of an endosymbiotic origin of mitochondria may still be unclear (Martin, 2007). However, in the early

1920s, Ivan Wallin put forward a less equivocal proposal for the endosymbiotic origin of mitochondria. His experiments challenged the validity of the past "microchemical" and staining methods used by other scientists to establish a cytoplasmic origin for this organelle, and provided one of the first pieces of evidence in support of the assertion of a bacterial nature of mitochondria (Wallin, 1923).

Until the discovery of mitochondrial DNA (mtDNA) (Nass and Nass, 1963), however, pioneer scientists in endosymbiosis were disregarded and their conclusions displaced by other theories. A predominant theory at the time was that all structural elements of the eukaryotic cell evolved sequentially, in one lineage. Also, it was generally assumed that mitochondria are synthesised *de novo* as differentiated compartments within a wholly autogenous eukaryotic cell. Also called "direct filiation", this idea was critically and extensively reviewed by Lynn Margulis in the seventies and early eighties (Margulis, 1970; Margulis, 1981). The scientific community seems to have remained sceptical until 1967 when Lynn Sagan (née Margulis) wrote a synthesis of relevant observations supporting the endosymbiotic origin of mitochondria and chloroplasts (Sagan, 1967). A milestone, this work provided an alternative to "direct filiation" by reviewing properties that mitochondria and chloroplasts have in common with modern bacteria, such as 70 S ribosomes, circular DNA and reproduction by binary fission. Undoubtedly, this period was a scientific revolution and the beginning of a new paradigm in organelle biology.

Following evidence that mitochondria contained DNA (Luck and Reich, 1964; Nass and Nass, 1963), the first mitochondrial genomes were sequenced (Anderson, et al., 1981). Initial analysis of mtDNA sequence and expression found unmistakable evidence that contemporary mitochondria were once free-living bacteria (Gray and Doolittle, 1982). Furthermore, it has also been agreed that mitochondria are closely related to the contemporary free-living bacteria (Yang, et al., 1985). However, even though studies on mtDNA have significantly increased our understanding on the evolutionary aspects of this organelle, many other questions have arisen within and alongside the endosymbiosis theory. Which bacterial species is the closest candidate to the proposed mitochondrial ancestor? What was the

evolutionary driving force for this endosymbiotic event to happen and to be maintained over billions of years? Even though the answers to these questions remain uncertain, several compelling studies have formulated assumptions worth describing.

1.1.2. Mitochondrial ancestry

Studies in the 1990s led to the suggestion that the α-proteobacterial order Rickettsiales contains most characteristics shared with the putative mitochondrial ancestor (Gray, 1998). An "Ox-Tox" model (Kurland and Andersson, 2000), whereby the proto-mitochondrion served to quench oxygen free radicals fitted nicely with the strict aerobic nature of *Rickettsia* species. The main thrust of this theory was that the acquisition of oxygen tolerance, at a time when atmospheric oxygen concentration was rising due to photosynthetic activity, was the most valuable advantage for a strict anaerobic host cell to acquire from an aerobic symbiotic organism (such as *Rickettsia prowazekii*). Additionally, the "Ox-Tox" theory propounds the importance of the origin of an ADP/ATP mitochondrial translocator, which made the exchange of energetic currencies between the symbiont and the host cell possible. *Rickettsia*'s ADP/ATP translocator, however, is unrelated to the mitochondrial one (Winkler and Neuhaus, 1999), partly undermining the theory that *Rickettsia* is related to the mitochondrial endosymbiont.

A large-scale analysis to assess the contribution of the mitochondrial endosymbiont to eukaryote nuclear genomes indicates the massive effect of endosymbiotic gene transfer on overall eukaryotic evolution (Esser, et al., 2004). This work also indicated the likelihood of other, more biochemically versatile, α-proteobacteria being good candidates for the mitochondrial endosymbiont. However, exactly pinpointing the mitochondrial endosymbiont to an extant α-proteobacterium is complicated by the dynamic nature of prokaryotic genomes due to lateral gene transfer and gene loss (Esser, et al., 2007). Nonetheless, biochemically more versatile α-proteobacteria such as *Rhodobacter* seem often to come to the fore (Atteia, et al., 2009).

1.1.3. Mitosomes and hydrogenosomes

There is currently no evidence that mitochondria-free eukaryotes ever existed. Supposedly "primitive" eukaryotes thought to be devoid of mitochondria (Cavalier-Smith, 1983) have been shown to be secondarily derived, having lost mitochondria. Eukaryotes such as *Microsporidium*, *Giardia*, *Trichomonas* and *Entamoeba* were put forward as related to the putative host to the mitochondrial endosymbiont because of their simple cell structures. Initial molecular phylogenies indeed placed these organisms (except *Entamoeba*) at the base of the eukaryotes (Sogin, et al., 1989). Subsequent work showed that these early phylogenetic reconstructions were fraught with methodological artefacts such as long-branch attraction (Brinkmann, et al., 2005), and the true relationships within the eukaryotes are currently not certain (Simpson and Roger, 2004). More importantly, the assumption that these protists were devoid of mitochondria was proved to be unfounded, as even if highly modified, mitochondria are found in all known protists (Lloyd, 2004). These specific protists are, however, devoid of "classic" 2 μm oval cristate mitochondria as their mitochondria turned out to be very small non-descript vesicles. The notion that these "primitive" eukaryotes were not that primitive after all became apparent when genes encoding typical mitochondrial proteins, such as chaperonins, were found in these organisms (Clark and Roger, 1995). For *Entamoeba*, antibodies raised against these proteins localized in a punctuate pattern throughout the cytoplasm suggestive of an organellar localization (Mai, et al., 1999). In addition, immunogold electron microscopy clearly labeled small organelles that had two membranes for *Giardia* and *Microsporidia* (Tovar, et al., 2003).

The presence of two surrounding membranes is a defining feature of organelles of endosymbiotic origin (Henze and Martin, 2003). These organelles of *Entamoeba*, *Giardia* and *Microsporidia* were termed mitosomes. Subsequent genome projects and large-scale proteomics attempts to elucidate the nature of these elusive mitochondria have not been able to provide much information about the role these organelles play. A common feature seems to be the production of iron–sulphur clusters as in other mitochondria (Lill and Mühlenhoff, 2005). Mitosomes do not

seem to play a role in ATP production and are devoid of components of the mitochondrial electron transport chain. No organellar genome has been detected, either directly (León-Avila and Tovar, 2004) or indirectly from the genome projects for these organisms (Clark, et al., 2007; Loftus, et al., 2005; Morrison, et al., 2007).

In the case of the trichomonads, the situation was slightly different, as an unusual organelle was known to be present for quite some time (Lindmark and Muller, 1973). This hydrogenosome had been shown to play a role in cellular energetics but unusually produced molecular hydrogen as a metabolic end product (Muller, 1993). Despite some initial claims (Cerkasovova, et al., 1976), no organellar genome could be detected in hydrogenosomes (Turner and Muller, 1983). Several mitochondrial proteins do, however, localize to these organelles (Bui, et al., 1996) and are targeted there by means of cleavable mitochondrial-like targeting signals (Bradley, et al., 1997). More recently, many more variations of hydrogenosomes have been discovered, as in the case of the diplomonad fish parasite *Spironucleus vortens* (Millet, et al., 2013). This parasite possesses double membrane-bound organelles with membrane potential, characteristic flavoprotein constituents, an Fe-hydrogenase, but are much smaller in comparison to conventional hydrogenosomes (Millet, et al., 2013).

The presence of hydrogenosomes and mitosomes as part of the mitochondrial family of organelles indicates a clear spread from simple metabolic organelles such as mitosomes that have lost their organellar genomes to classic textbook aerobic mitochondria. These novel organelles do also fit with the above-mentioned bioenergetic theory of eukaryotic origins (Lane and Martin, 2010; Martin and Muller, 1998). However, despite numerous attempts to determine the nature of the mitochondrial ancestor and the evolutionary driving force behind endosymbiosis, this topic is still open to debate.

1.1.4. The driving force behind endosymbiosis

The exact time is still debatable, but recent studies suggests that the Earth's atmosphere was completely anoxic during the so called "stage 1", which ranges from 3.85 to 2.45 Gyr (Holland, 2006). Molecular oxygen arose in Earth's atmosphere only approximately 2.5 Gyr ago, as a waste product of a recent innovation in autotrophic metabolism at that time, known as oxygenic photosynthesis. Thus, it is plausible to imagine that an anaerobic host cell would have benefited from the oxygen-reducing properties of a mitochondrial endosymbiont, especially after the abrupt built up of an oxidizing environment, as suggested before by (Kurland and Andersson, 2000). However, whether endosymbiosis occurred while the Earth was still anoxic or post photosynthetic oxygen evolution is still uncertain. Here I discuss two theories for the endosymbiotic origin of mitochondria under anoxic conditions.

Molecular data has revealed that hydrogenosomes and mitochondria might be closely related (Horner, et al., 1996). These findings enlightened a new theory for an anoxic origin of mitochondria (Martin and Muller, 1998). It posits that a syntrophic relationship between a strictly H_2-dependent host methanogenic cell and a hydrogenosome-like symbiont might have been the driving force for such event. In a situation where the geological supply of H_2 was interrupted, the endosymbiont may have satisfied the needs of the host cell for molecular hydrogen, forcing those two cells to be permanently bound in an obligatory endosymbiotic relationship (Martin and Muller, 1998) (Figure 1.1). However, the fact that modern eukaryotes do not possess methanogenic genes exposes what could be a flaw of the hydrogenosome theory for the origin of mitochondria. Moreover, hydrogenosomes are more likely to be convergent adaptations of mitochondria to anaerobic environments, rather than the ancestral organelle (Hackstein, et al., 1999).

Another possibility was that, instead of molecular hydrogen (H_2), hydrogen sulphide (H_2S) might have been the molecule that created this inter-dependent relationship between host and symbiont. This hypothesis, named "sulphur syntrophy" (Searcy, 2003), is based on experimental results demonstrating that some

archeal species have the capacity to produce H_2S, and that purple-sulphur bacteria are capable of oxidising the same compound. In this case, H_2S would act as an electron shuttle carrying reducing equivalents to the mitochondrion and returns oxidised equivalents back to the cytoplasm (Searcy, 2003). The occurrences of such H_2S-based redox reactions can be often seen nowadays in many anoxic environments, including hydrothermal vents (reviewed by (Lloyd, 2006).

Concomitant with the endosymbiotic event, eukaryotes arose from the prokaryotes. This is thought to have happened only once in life's history, and it is proposed that mitochondria have played an indispensable role in this evolutionary process (Lane and Martin, 2010). According to this hypothesis, the ATP produced by mitochondrial oxidative phosphorylation has provided the energy necessary for the expression of an immensely larger number of genes than would have been possible without such a powerhouse. The endosymbiotic event that resulted in the establishment of the mitochondrion was therefore a crucial event for the evolution of the eukaryotes as a whole. The opposite is that the evolution of a complex eukaryote enabled the endosymbiotic event that led to the establishment of the mitochondrion, is untenable according to the rules of bioenergetics (Lane and Martin, 2010).

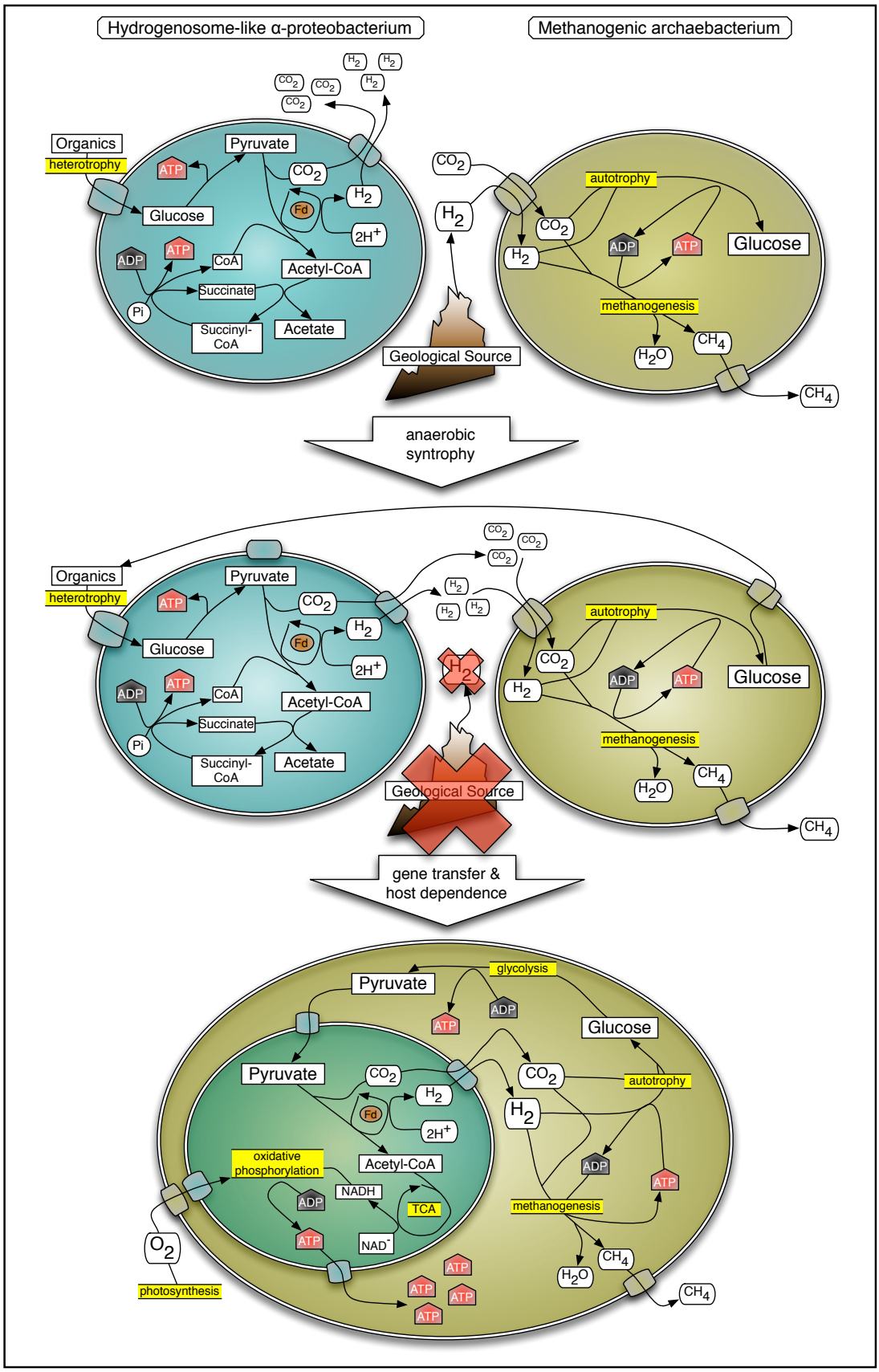

Figure 1.1. The hydrogen hypothesis for the origin of mitochondria. Adapted from (Martin and Muller, 1998).

1.1.5. Why do organelles retain a small subset of genes?

Many mitochondrial and α-proteobacterial genomes have been sequenced in the past decades. Comparative genomic analysis clearly shows that modern mitochondrial genomes are severely reduced compared to those from α-proteobacteria. This has been caused by gene loss, but most importantly, because the mitochondrial genes have been functionally transferred to the nucleus over time (Race, et al., 1999; Timmis, et al., 2004). This process was named endosymbiotic gene transfer (Martin, et al., 2001) and is a special case of lateral gene transfer. As this was not an instantaneous event but something that happened over time, each and every lineage has transferred and lost genes at his or her own pace. As a result of this, a large variety of mitochondrial and chloroplast genome sizes and genome contents can be found. An interesting example is the causative agent of malaria *Plasmodium falciparum*, which is known to possess one of the smallest mitochondrial genomes with only 5,967 base pairs (bp). This very small genome, nonetheless, still contains three core genes encoding the cytochrome b (*cob*) and cytochrome oxidase subunits I (*cox1*), and III (*cox3*) of the respiratory electron transport chain (Omori, et al., 2007). On the other hand, the gene-rich mitochondrial genome of *Reclinomonas americana* is comprised of 69,034 bp and 67 protein-coding genes (Lang, et al., 1997). Although there are plenty of notable studies unveiling the mechanisms and forces driving the lateral gene transfer to happen, this subchapter aims to canvass the other side of the coin: Why is a small subset of genes always kept in the organellar genome of contemporary eukaryotes?

There are several hypotheses attempting to explain the selective pressure that maintains genomes in mitochondria and chloroplasts. The hydrophobicity hypothesis suggests that certain organellar genes encode hydrophobic proteins that may be problematic for cellular targeting machineries, which may cause them to be mistargeted to the endoplasmic reticulum (von Heijne, 1986). Support for this hypothesis comes from the observation that *cox1* and *cob* genes are present in every mitochondrial genome sequenced so far (excluding the organelles of *Blastocystis* (Perez-Brocal and Clark, 2008), which are intermediates between mitochondria and

hydrogenosomes, and *Nyctotherus ovalis* (van Hoek, et al., 2000)), and the respective proteins encoded by these genes are classified as typically hydrophobic peptides. Moreover, experimental analysis has shown that cytosolically synthesized apocytochrome *b* in yeast is not properly imported into mitochondria (Claros, et al., 1995). In plants, a similar experiment reported that the *in vitro* synthesised COX2 protein is unable to be imported into soybean mitochondria, unless one of the transmembrane domains is removed and a few critical amino acid changes are made (Daley, et al., 2002). Although these data have upheld the hydrophobicity hypothesis for some genes, this hypothesis remains unable to explain various other cases. For example, the mitochondrial ADP/ATP translocator (Adrian, et al., 1986) is almost completely buried in the mitochondrial inner membrane and is very hydrophobic but is, nonetheless, always nuclear encoded. It might be argued that these are eukaryotic inventions and would therefore never have been mitochondrially encoded in the first place. Even if this is true, the cell has no problem in targeting this highly hydrophobic protein, containing at least six membrane-spanning helices, to the correct cellular compartment. Another theory seeking to explain the presence of organellar genomes states that some organellar genes have an idiosyncratic codon usage, which would preclude their nuclear expression, therefore locking them into the organelles (Doolittle, 1998). In contrast, it has been shown in tobacco that the chloroplast gene encoding the large subunit of Rubisco (*rbcL*) can be expressed in the nuclear genome of tobacco if relocated (Kanevski and Maliga, 1994), therefore exposing a drawback on the idiosyncratic codon usage hypothesis.

Based on previous studies that had shown that gene expression was controlled by the redox state in bacteria a hypothesis was put forward (Allen, 1993). This hypothesis suggests that mitochondria and chloroplasts have kept some specific genes in their genomes in order to enable an *in situ* redox regulation of their expression. These specific genes are thought to encode either the respiratory core subunits in the mitochondria, or the photosynthetic apparatus core subunits in the chloroplasts. In other words, if they were relocated to the nucleus, direct transcriptional regulation of these genes by the organellar redox state would not be possible. This hypothesis was named the CoRR hypothesis, which stands for Co-location for Redox Regulation

(Allen, 2003). According to this hypothesis, there are two main players participating in the regulation of redox-driven gene expression: a redox sensor and a redox response regulator. The redox sensor is thought to be an electron-carrier that initiates control of gene expression upon oxidation or reduction. The redox response regulator, on the other hand, is proposed to be a DNA-binding protein that modifies gene expression as a result of the action of the redox sensor (Figure 1.2).

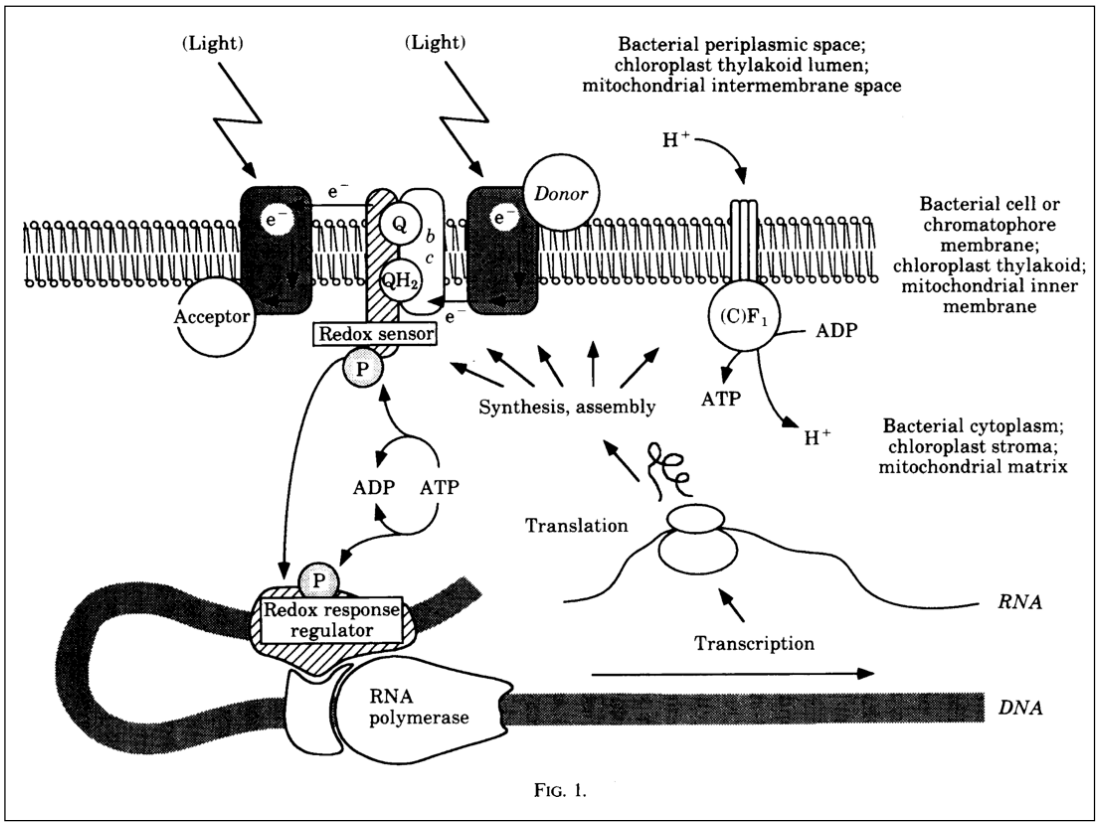

Figure 1.2. A proposed two-component model of redox regulation of transcription in mitochondria and chloroplasts (Allen, 1993).

Reactive oxygen species (ROS) result from excitation or incomplete reduction of molecular oxygen, and are unwelcome harmful by-products of normal cellular metabolism in aerobic organisms (Chance, et al., 1979; Naqui, et al., 1986). In most eukaryotes, mitochondrial electron transport is the major generator of ROS (Chen, et al., 2003). It is extremely important for the cell to keep the ROS production under control to avoid cell damage (Jo, et al., 2001) as this can eventually lead to the initiation of programmed cell death (PCD) (Gechev, et al., 2006; Jacobson, 1996). The CoRR hypothesis suggests that by regulating the individual expression of genes encoding core subunits of the electron transport chain in mitochondria, and the

photosystem I and II core subunits in chloroplasts, ROS production can be controlled. Light quality and quantity are known to influence the plastoquinone pool, and hence the redox state of chloroplasts (Allen, et al., 1995). Indeed, it has been shown that changes in the redox status of the plastoquinone pool by variation of light quality do control the rate of transcription of genes encoding reaction-centre apoproteins of photosystem I and II (Pfannschmidt, et al., 1999). It was predicted that a redox sensor protein would control this switch. In 2008, this sensor protein was identified in *Arabidopsis thaliana* (Puthiyaveetil, et al., 2008). It was termed chloroplast sensor kinase or CSK. Phylogenetic analysis shows that the plant CSK shares common ancestry with cyanobacterial histidine kinases, suggesting that photosynthetic CoRR regulation has been present since chloroplasts were still free-living cyanobacteria. Furthermore, recent studies on *A. thaliana* CSK have also indicated that specific cysteine residues are well conserved between cyanobacteria and higher plants. These are thought to be crucial for sensing the redox state of the chloroplast plastoquinone pool (Ibrahim, 2009; Puthiyaveetil, et al., 2010). Thiol-based regulatory switches involving cysteine residues are known to play central roles in cellular responses to oxidative stress (Paget and Buttner, 2003). For example, *Escherichia coli* ArcB is a sensor kinase that contains redox-active cysteine residues, and, upon changes in redox states of the quinone and menaquinone pools, regulates the transcription of genes for aerobic and anaerobic metabolism (Bekker, et al., 2010). Whether mitochondria have a bacterial-type redox sensor kinase that regulates transcription is still unknown. With exception of the protist order Jakobids (i.e. *Reclinomonas americana* (Lang, et al., 1997)), mitochondria have lost their bacterial RNA polymerase and sigma factors, retaining only a phage-type transcriptional machinery (Hedtke, et al., 1997).

Few relevant studies have been carried out to understand the role of the mitochondrial redox state on the regulation of organellar gene expression. One such study describes the incorporation of ^{35}S-methionine into newly synthesised mitochondrial proteins in relation to the redox status of the electron transport chain (ETC) ubiquinone pool (Allen, et al., 1995). By the use of inhibitors of specific sites of the ETC, it was reported that protein synthesis was precluded by inhibitors of

ubiquinone reduction, but not by inhibitors of ubiquinol oxidation. Furthermore, it was found that electron transport through succinate:ubiquinone oxidoreductase (Complex II) was specifically required for protein synthesis, strongly suggesting that a subunit of complex II, or a component closely associated with this complex, is involved in a regulatory system that couples electron transport to protein synthesis (Escobar Galvis, et al., 1998). Another study using *Solanum tuberosum* (potato) mitochondria investigated the role of a variety of electron transport inhibitors on organellar RNA synthesis. It was found that the redox state of the Rieske iron-sulphur protein was the major determinant of organellar RNA synthesis (Wilson, et al., 1996). RNA synthesis was positively affected by inhibitors that act on the substrate side of the Rieske iron–sulphur protein. These inhibitors cause oxidation on the oxygen side of their site of action. On the other hand, if inhibitors were used that reduce the substrate site, then RNA synthesis was decreased. Redox regulation of plant mitochondrial glutamate dehydrogenase (Tarasenko, et al., 2009) and DNA topoisomerase (Konstantinov, et al., 2001) have also been reported. It has been suggested that this plays a role in coupling respiratory electron transport with mitochondrial gene expression.

1.2. Mitochondrial free radical theory of ageing

As discussed in the previous section, mtDNA is retained in the mitochondrial matrix, where aerobic respiration takes place, and highly reactive oxygen species (ROS) are generated as a by-product. According to the mitochondrial theory of ageing (MTA), there is a penalty for keeping both, mtDNA and the respiratory apparatus, in the same compartment: we get old and die. However, since the MTA was first proposed, studies have shown that some of the original predictions are not true, while others have been shown to be valid. Nevertheless, while many predictions of the theory still remain to be empirically tested, intense debate takes place in the field. In light of recent advancements, this Sub-chapter discusses how some ROS are produced, and their implications in cellular (and mitochondrial) deterioration processes that may contribute to ageing.

1.2.1. Reactive oxygen species (ROS) and oxygen free radicals

For the past half century, ROS generated by biochemical processes are argued to be the central molecule involved in the decay of cellular components observed in ageing processes. It is important, however, to note that not all ROS are oxygen free radicals, and vice versa. Hydrogen peroxide (H_2O_2) itself is not a free radical as it does not contain any unpaired electrons, but it is still considered a ROS of biological relevance. Similarly, molecular oxygen (O_2) is an example of a stable free radical that is not reactive, because although individual oxygen atoms have only six valence electrons, it would prefer to have eight (the octet rule). From here on, only the aspects of ROS of biological importance will be discussed in this thesis.

In aerobic eukaryotes, the mitochondrial respiratory electron transport chain is one of the main sites of ROS production in the cell. Although molecular O_2 reducing reactions can also occur in other compartments of the cell (i.e. Fenton reaction (Goldstein, et al., 1993), or cytosolic NADPH oxidase (Dikalov, 2011)), this section discusses mainly the mitochondrial sources of ROS production.

Molecular O_2 was probably chosen by nature as the final electron acceptor of respiration, instead of halogens for instance, because of its physical state, satisfactory solubility in water and suitable combination of kinetic and thermodynamic properties (Naqui, et al., 1986). These properties are mostly attributed to the fact that O_2 is in a "triple ground state", with two unpaired electrons with the same spin number. This spin restriction makes the uptake of single electrons by molecular O_2 thermodynamically unfavourable (Table 1), unless it interacts with another paramagnetic center to engage in exchange coupling (i.e. transition metal ions). As a result, enzymes such as bacterial and mitochondrial cytochrome c oxidase (complex IV) may have evolved the capacity of binding tightly to molecular O_2 and transition metals, and probably specialised in two-electron transfers to overcome such a thermodynamic barrier (Chance, et al., 1975).

Table 1.1. Thermodynamic parameters for the reduction of molecular O_2 to H_2O (extracted from (Naqui, et al., 1986))

Reaction(s)	E_0 (pH 7), V
Four-electron transfer	
$O_2 + 4H^+ + 4e^- \rightleftharpoons 2H_2O$	+ 0.82
Two two-electron transfer	
$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O_2$	+ 0.3
$H_2O_2 + 2H^+ + 2e^- \rightleftharpoons 2H_2O$	+1.35
Four one-electron transfer	
$O_2 + e^- \rightleftharpoons O_2^{\bullet -}$	-0.33
$O_2^{\bullet -} + e^- + 2H^+ \rightleftharpoons H_2O_2$	+0.94
$H_2O_2 + H^+ + e^- \rightleftharpoons H_2O + {}^{\bullet}OH$	+0.38
${}^{\bullet}OH + H^+ + e^- \rightleftharpoons H_2O$	+2.33

Other studies suggest that superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals (${}^{\bullet}OH$) are produced by NADH:ubiquinone oxidoreductase, or complex I (Kussmaul and Hirst, 2006; Ohnishi, et al., 2010) and cytochrome c reductase, or complex III (St-Pierre, et al., 2002) – where large changes in the potential energy of the electrons, relative to the reduction of oxygen, occur. Highly unstable reduced

ubisemiquinone ($Q^{-\bullet}$) is generated as a result of those abrupt potential changes in complexes I and III, which may, in turn, be the cause of single electrons transfers to molecular O_2 yielding $O_2^{\bullet-}$ and $^{\bullet}OH$.

1.2.2. Mitochondrial theory of ageing

Denham Harman, an American gerontologist, was the first one to propose that the accumulation of oxygen free radicals is the cause for ageing in living organisms (Harman, 1956). This idea became known as the Free Radical Theory of Ageing (FRTA). Surprisingly, at the time he proposed the FRTA, Harman did not know that oxygen free radicals were indeed produced in the cell by a variety of biochemical reactions. Only later, following advancements in the free radical biology, it was empirically demonstrated that those compounds were produced in the cell, mainly as consequence of energy production metabolism such as aerobic respiration.

Combining the discovery of mtDNA with the FRTA, Harman proposed yet another theory, called the mitochondrial theory of ageing (MTA) (Harman, 1972). This theory states that superoxide anions ($O_2^{\bullet-}$) produced by accidental single-electron transfers in the mitochondrial respiratory ETC reacts with the mtDNA retained in the matrix, causing deleterious rearrangements that accumulate over time. As a result, more defective ETC subunits are encoded by the mutated mtDNA, which in turn causes more accidental single-electron transfers, which cause more mutations in the mtDNA, and so on. This "vicious circle" was deemed to be the main cause of ageing in aerobic organisms (Harman, 1972). Indeed, not only $O_2^{\bullet-}$ has been shown to be potentially hazardous ROS, but also H_2O_2 and $^{\bullet}OH$ have been shown to readily damage lipids, proteins and nucleic acids present in the same compartment (Ames, et al., 1993; Ames, et al., 1995; Shigenaga, et al., 1994).

Whether ROS are the direct cause of such decline of mitochondrial function seen in ageing, or there is merely a strong correlation, is still under debate (Afanas'ev, 2010; Jacobs, 2003). Some studies suggested that ageing-related mtDNA

rearrangements might arise from mtDNA replication errors rather than ROS-induced damage (Park and Larsson, 2011), which may subsequently trigger downstream apoptotic markers rather than more ROS production (Kujoth, et al., 2005). Another line of work proposes that ageing processes induced by mtDNA mutations are not at all involved in ROS generation (Trifunovic, et al., 2005; Trifunovic, et al., 2004).

However, more recent studies seem to support most predictions of the MTA. Using a superoxide-dependent chemiluminescent assay, a study found that the superoxide production increases with age in brain tissues of three different organisms: a senescence-accelerated mice line, Wistar rats and pigeons (Sasaki, et al., 2008). Moreover, (Miyazawa, et al., 2009) showed that oxidative damage and excess apoptosis occur with ageing in the brain, oculus and kidney of mice – in correlation with $O_2^{\bullet -}$ concentrations – but comparatively, lower concentrations were found in cardiac and muscle tissues. Additionally, it has been reported that pressure-induced arterial $O_2^{\bullet -}$ concentrations increase as a function of age (Jacobson, et al., 2007), and that endogenous extracellular superoxide dismutase (ecSOD) plays an important role in protection against endothelial dysfunction during aging in mice (Lund, et al., 2009). Complementarily, another group found that, compared to the wild-type mice, the long-lived Ames dwarf mice have much lower quantities of F2-isoprostanes (IsoPs), which are products of lipid peroxidation and used as markers for oxidative stress (Choksi, et al., 2007). In humans, there were also detected a significant age-related increase of lipoperoxides, and decrease of glutathione peroxidase activity and amount of antioxidants in the plasma serum (Mendoza-Nunez, et al., 2007). In addition, two studies provide direct support for the hypothesis that endothelial oxidative stress develops with ageing in healthy men and is related to impaired vascular endothelial function (Donato, et al., 2007; Rodriguez-Manas, et al., 2009).

1.2.3. ROS scavenger enzymes

In order to reduce the detrimental effects of ROS in the cell, most eukaryotes have evolved specialized ROS scavenger enzymes that are capable of reducing ROS a step further towards water. Two classes of such proteins are discussed here: superoxide dismutases and catalases. Superoxide dismutases (SODs) are usually found in three different isoforms in eukaryotes: Fe-SOD, Mn-SOD and CuZn-SOD. The first two are thought to share common ancestry, but have no homology with CuZn-SOD – indicating that the latter may have originated from an independent lineage (Grace, 1990). SOD activity was first characterised by (McCord and Fridovich, 1969), and it was found that this protein is capable of reducing $O_2^{\bullet-}$ one step further to generate H_2O_2. Although it was initially reported that overexpression of SODs may increase the life span of fruit flies (Parkes, et al., 1998), more recent studies have demonstrated that SOD is dispensable for a normal lifespan in animals (Van Raamsdonk and Hekimi, 2009; Van Raamsdonk and Hekimi, 2012).

Catalases are another example of ROS scavenging enzymes. They are ubiquitous and have been extensively studied for over a century. A variety of catalase isoforms are virtually present in all eukaryotes, where they catalyses the reduction of H_2O_2 into molecular O_2 and water (Alfonso-Prieto, et al., 2009). The crystal structure of catalase was first determined in the early eighties (Murthy, et al., 1981), and an iron-heme was identified as the main ligand in the active site. Contrary to SODs, catalases have been shown, to a certain extent, to be more promising in preventing ageing-related degeneration caused by ROS in animals (Bai, et al., 1999; Lee, et al., 2010).

Catalase and SODs clearly are important enzymes that protect cellular components against oxidative damage. However, their activity seems not to be sufficient to completely prevent or revert ageing.

1.3. The evolution of sexes and uniparental inheritance

Many theories have been put forward to explain the origin and maintenance of the two separate sexes, or anisogamy, as we know them in multicellular eukaryotes. Most of those theories find support for their predictions in theoretical population genetic and mathematical models. Other theories are underpinned by observations of the biochemistry of the cell, algal gamete differentiation studies, or even on the implications of efficiency in finding a mating partner by using sexual pheromones. These fronts are discussed in this sub-chapter.

1.3.1. Earlier proposals

Earlier classical models have focused on the availability of nutrients as the main selective pressure. However, given that a fixed amount of material is necessary for zygote development, natural selection would certainly drive the gametes to be either small sperm-like gametes produced in high quantities, or large egg-like nutrient-storing gametes produced in small quantities, thus failing to predict sexual dimorphism as an outcome. Only when dominant Mendelian inheritance of gamete size was included into the equations, the establishment sexual dimorphism could then be predicted (Parker, et al., 1972). This same work has also proposed that isogametic fusion between two egg-type gametes was initially favoured, but due to supposedly faster adaptation rates in sperms or instability of isogametic populations, this disruptive selection lead to the evolution and maintenance of asymetric fusions (Parker, et al., 1972). Further observations about gamete differentiation in the green algae order Volvocales identified a pattern, in which increased vegetative size and complexity should be accompanied by the development of anisogamy (Bell, 1978; Knowlton, 1974). Although it is seen that morphological complexity often accompanies increased gamete dimorphism, it does not reliably predict whether a given species will display gamete dimorphism (Madsen and Waller, 1983).

1.3.2. Intragenomic conflict theories

A caveat of the earlier proposals is that, due to the little information known on mitochondrial and chloroplast genomes at that time, cytoplasmic genetic inheritance was not taken into consideration. For the first time, the psychologist Leda Cosmides and her husband, the anthropologist John Tooby, introduced the cytoplasmic genetic inheritance "variable" into consideration (Cosmides and Tooby, 1981). Antagonistic to views favouring gene cooperation and specialization for an increase in efficiency (Maynard Smith, 1982), Cosmides and Tooby (1981) posited that the fitness of cytoplasmic genetic sets is increased at the nuclear genome's expense in sort of intracellular warfare termed "intragenomic conflict". Consequently, the evolution of maternal cytoplasmic inheritance and anisogamy is suggested to have minimized the negative effects of such a quarrelsome co-evolution process, by means of allowing only one side to contribute to the majority of traits of the developing zygote (Cosmides and Tooby, 1981).

The intragenomic conflict was further developed into mathematical models based on populational genetic studies (Hurst and Hamilton, 1992). Their model shows that, if sexual dimorphism requires gametic fusion, then nuclear genes should evolve to prevent conflict between cytoplasmic genes. In turn, these genes would give rise to an incompatibility system that prevents inbreeding and maximize the number of potential reproductive mates (Hurst and Hamilton, 1992). More recently, it was suggested that each taxon should be studied and analysed independently, and that a general solution for origin of sexual dimorphism could not be reached by mathematical modelling studies (Randerson and Hurst, 2001).

1.3.3. Mito-nuclear co-adaptation hypothesis

Recently proposed, the mito-nuclear co-adaptation hypothesis combines intragenomic conflict views with free radical biology (Lane, 2011). Because most of mitochondrial genes required for oxidative phosphorylation have been transferred to

the nuclear genome, eukaryotes now possess a mosaic electron transport chain that contains a mix of nuclear-encoded and mitochondrially-encoded electron transport protein subunits. As a consequence, adequate matching between those subunits is required for optimal respiratory activity in mitochondria. Mismatches between those subunits are thought to lead to poor respiratory protein complexes assembly, which in turn lowers the fitness, and generates ROS leak. According to the mito-nuclear co-adaptation hypothesis, the evolution of two separate sexes and uniparental inheritance facilitates the selection for matching cytoplasmic and nuclear genomes, by means of reducing the occurence of mitochondrial heteroplasmy (Lane, 2011).

The effects of mito-nuclear genetic mismatch, and its relationship with the origin and maintenance of two separate sexes were later investigated using mathematical models (Hadjivasiliou, et al., 2012). This study considered a single nuclear gene that interacts with a single mitochondrial gene throughout a given cycle of five steps: mutation, selection, bottleneck, meiosis and syngamy. Their results suggest that uniparental inheritance and mitochondrial genetic bottleneck are positive factors that contributed to the population fitness, and that this selective pressure for mito-nuclear co-adaptation may have favoured the evolution of two sexes (Hadjivasiliou, et al., 2012).

1.3.4. The mitochondrial genetic bottleneck theory

Although the mitochondrial genetic bottleneck theory does not attempt to explain the origin of uniparental inheritance or separate sexes, it aims to solve the previous question of how mtDNA is transmitted as an accurate template between generations? In other words, how is it that accumulated mutations that arose with ageing are not passed on to the next generation. The theory posits that in primordial germ cells (PGCs), prior to the oogenesis, the number of maternal mtDNA molecules is drastically decreased causing a genetic bottleneck effect as seen also in populational studies (Samuels, et al., 2010). In turn, this causes a decrease in mitochondrial heteroplasmy, which may be beneficial to the developing embryo

(Samuels, et al., 2010). However, some studies conclude that no such reduction of mtDNA molecules occurs at the PGCs (Cao, et al., 2007; Cao, et al., 2009), and other studies show contrary results on mtDNA variance levels between oocytes and developing embryos (Jenuth, et al., 1996; Wai, et al., 2008).

In congruence with the bottleneck theory, other studies have proposed the existence of a "purifying sieve" in the female germ line, which discriminates between healthy and unhealthy mitochondria, allowing only the healthy ones to populate the offspring. These conclusions are either based on results showing the elimination of a severe mtDNA mutation after a number of generations (Fan, et al., 2008), or based on low nonsynonymous/synonymous substitution (*d*N/*d*S) ratios found in mtDNA sequences over generations (Stewart, et al., 2008). However, the mechanisms by which the proposed processes occur are still unknown.

1.3.5. The protected female germ line mitochondria

If the MTA is correct, important questions remain to be answered: How is mtDNA transmitted as an accurate template between generations? Why is the somatic mitochondrial degeneration acquired over a life span not passed on to the offspring? Ultimately, why is age not inherited?

A hypothesis put forward in 1996 by John F. Allen (Allen, 1996) proposes an elegant explanation for how that happens – the combination of uniparental inheritance with a division of labour between male and female gametes is the key for resetting the ageing clock every new generation. According to (Allen, 1996), female gametes, which are immotile, carry a special population of mitochondria that are exempted from the ageing processes proposed by the MTA. These mitochondria, denominated "template mitochondria", suppress oxidative phosphorylation to cease ROS generation in the respiratory ETC, and as a result, their mtDNA is protected against deleterious rearrangements caused by those mutagenic ROS. If this theory is correct, this means that oocytes carry "immortal" mitochondria that never age. Male gametes, on the other hand, are motile and depend on respiration to produce large amounts of ATP and find the non-motile female counterpart. That implies that sperms contain disposable mitochondria, which is *de facto* observed in nature – uniparental inheritance is the rule for almost all eukaryotes. In this scenario, uniparental inheritance, separate sexes, and anisogamy, may have evolved simply to solve the age inheritance problem that arose with aerobic respiration and mtDNA retention in eukaryotes (Allen, 1996).

An advantage of (Allen, 1996) is that it makes many testable predictions. First, for the female germ line template mitochondria to remain quiescent throughout development, they should repress their bioenergetic function by the absence of cytochrome or other electron transport complexes. This is suggested to occur primarily at the level of transcription of the mitochondrial-encoded genes. Some empirical studies seem to support this first prediction. In *Xenopus laevis*, there has been identified an mtDNA-binding protein abundant in the oocytes, which

inhibits the transcription of mitochondrial genes (Barat-Gueride, et al., 1989; Ghrir, et al., 1991). More recently, another work reported low quantities of ATP synthase proteins and ROS in *Xenopus* oocytes. They explicitly suggest that *Xenopus* oocytes contain suppressed mitochondria in order to support the accurate transmission of DNA to the next generation (Kogo, et al., 2011), however, with no mention of this hypothesis proposed by Allen. In mouse, it has been previously shown that the mitochondrial genome is largely inactive in the egg and two-cell embryo stages, and that mitochondrial transcription is only initiated during cleavage – when structural and functional differentiation of mitochondria also occurs (Piko and Taylor, 1987; Taylor and Piko, 1995).

Another prediction of (Allen, 1996) is that in newly developing female, template mitochondria acquired from the mother's oocyte are sequestered by a small number of female germ line precursor cells at an early stage in development, and those will subsequently form the new female germ line pool of template mitochondria (Figure 1.3). This process is then repeated indefinitely, thus guaranteeing a batch of fresh mitochondria for each ensuing generation. In the case of male gametes and somatic mitochondria of both sexes, template mitochondria would then differentiate into active mitochondria that are capable of oxidative phosphorylation and ATP production, hence providing all the energy necessary for living (Figure 1.3). However, it is also suggested that, once template mitochondria differentiate into active ones, the ageing clock starts ticking, and this step is irreversible – in other words, the hypothesis predicts that rewinding the ageing clock to the past is a forbidden process. Although little evidence exists to support this prediction, it has been observed that two populations of morphologically distinct mitochondria are segregated in the oocytes of *Xenopus* (Tourte, et al., 1984), and only one of which contributes to the germ plasm (Mignotte, et al., 1987).

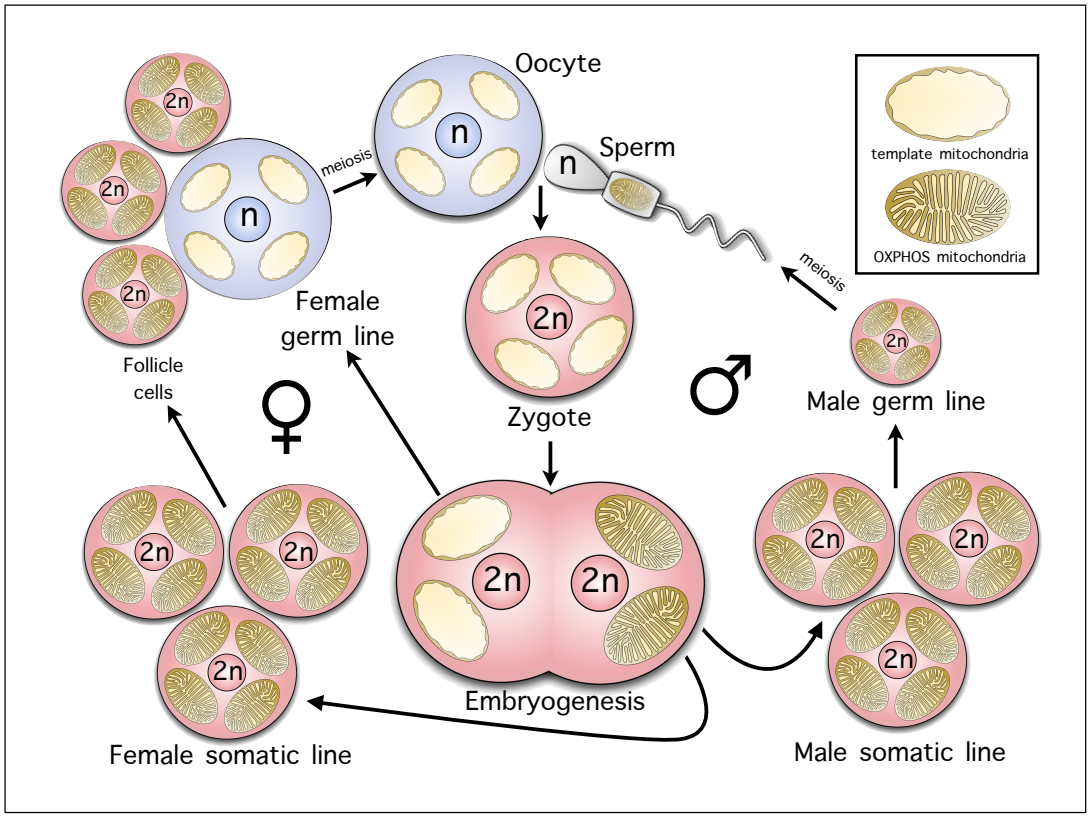

Figure 1.3. A model for maintenance and maternal inheritance of template mitochondria in eukaryotes. An oocyte contains a nucleus with a haploid chromosome number (n) and a cytoplasm with multiple template mitochondria. A sperm cell, also with a haploid nucleus (n), is motile, and its motility requires ATP from active mitochondria performing oxidative phosphorylation (OXPHOS). Following fertilization, active sperm mitochondria are rapidly degraded, leaving only the maternal, template mitochondria in the cytoplasm of the diploid (2n) zygote (or fertilized egg). Successive cell divisions in embryogenesis in involve mitosis and differentiation, and division, of most template mitochondria into active, OXPHOS mitochondria, which eventually dominate and populate not only somatic tissues but also the male germ line, from which sperm are generated by meiosis in males of the next generation. However, some cells are sequestered and continue to carry only quiescent, template mitochondria indefinitely, through meiosis and oogenesis to give the oocytes of females in the next generation. These cells comprise the female germ line. Female germ cells are never supplied with ATP by oxidative phosphorylation in their own mitochondria, but depend for their maintenance, at low metabolic rate, on ATP supplied, directly or indirectly, by neighbouring somatic cells (follicle cells or nurse cells) that are specially adapted for this role. Diagram adapted from (Allen, 1996).

Other studies of doubly uniparental inheritance of mtDNA (DUI) in freshwater mussels may have elucidated yet another line evidence supporting the theory proposed by (Allen, 1996). In these rare cases of DUI, such as in *Mytilus sp.*, paternal mitochondria survive in the male zygote, but not in the female one, where only maternal mitochondria are maintained (Zouros, et al., 1994). Additionally, in the particular cases of amitochondriate eukaryotes possessing mitosomes or hydrogenosomes, which do not carry their own DNA in most of cases (as discussed more in detail in Section 1.1.3), the selective pressure for the maintenance of two separate sexes and uniparental inheritance seems to be weaker, inasmuch as sexual reproduction still remains a mystery for many of those species (Ayala, 1998; Birky, 2009).

1.4. Background on the experimental systems

1.4.1. Jellyfish – *Aurelia aurita*

The genus *Aurelia* belongs to the class Scyphozoa within the phylum Cnidaria (Collins, et al., 2006). The Cnidaria are an ancient animal phylum, with the palaeontological record (Cartwright, et al., 2007) and evolutionary inference (Ojimi and Hidaka, 2010) suggesting an origin prior to Middle Cambrian period (Butterfield, 1997; Condon, et al., 2012) perhaps even during the late Proterozoic era, up to 700 million years ago (Butterfield, 2000), and before the emergence of the Bilateria (Extavour and Akam, 2003). Cnidaria are simple organisms consisting of an endoderm and ectoderm, between which is a largely acellular and watery 'mesogloea' that defines their gelatinous body. The Scyphozoa, or 'true' jellyfish, exhibit alternation of generations between a sexually reproductive phase, the medusa, an asexual phase, the polyp, and an intermediate stage called ephyra (Morandini and Da Silveira, 2001) (Figure 1.4a-b). The medusae are typically dioecious (Figure 1.4c-d).

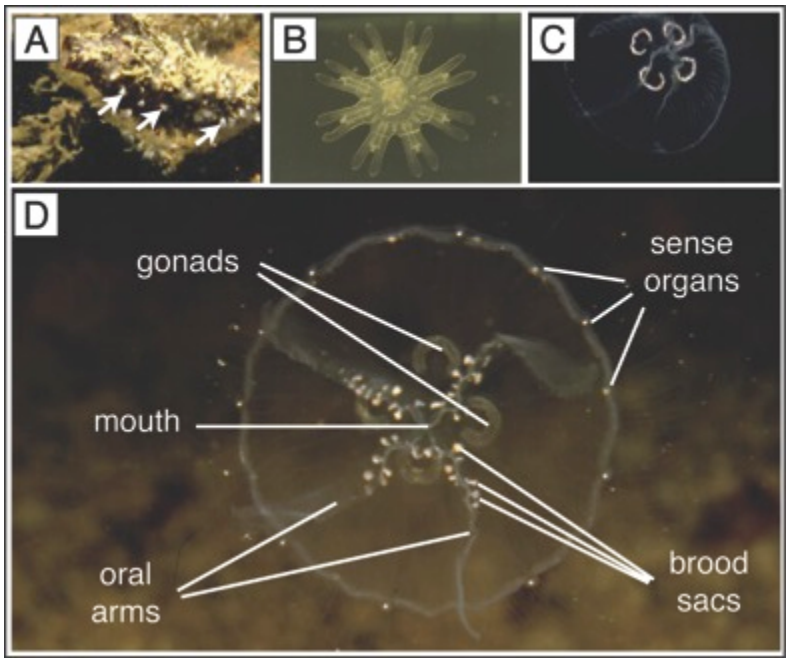

Figure 1.4. Different stages of *Aurelia aurita*: Polyps (white arrows) are the sessile asexual stage (a), which releases the intermediate motile ephyra (b), which in turn gives rise to the sexual stage dioecious medusa (c). Anatomy of *A. aurita* medusa; main organs are indicated in the image (d). Images kindly provided by Cathy Lucas, University of Southampton.

The genus *Aurelia* is found throughout the world's oceans (Dawson and Martin, 2001), with *Aurelia aurita* a very typical scyphozoan in terms of reproduction and development (Lucas, 2001). In wild populations, most medusae live for less than a year, dying following gamete release (Lucas, 2001). Horseshoe-shaped gonads are located within the gastric cavity (Figure 1.5). Fertilization is external, after which fertilized eggs transfer into brood sacs on oral arms hanging down below the umbrella where they develop into ciliated planula larvae. The mitochondrial genome of *Aurelia aurita* is composed of 13 energy transduction protein-coding genes, small and large subunit rRNAs, and methionine and tryptophan tRNAs (Shao, et al., 2006). As one of the most ancient dioecious metazoan ancestors alive, jellyfish can be considered an ideal model organism of choice for studying evolutionarily conserved elemental features, which may span the entire animal kingdom.

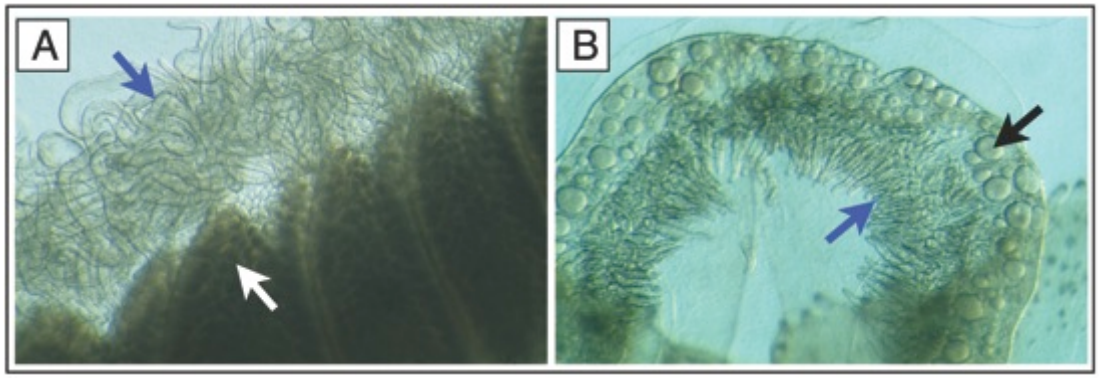

Figure 1.5. *Aurelia aurita* medusa gonads. Male medusa (a), female medusa (b). Blue arrows indicate the gastric filaments that surround the mouth. Black arrow points to an oocyte. White arrow points to sperm sacs, seen as darker spots. Images kindly provided by Cathy Lucas, University of Southampton.

1.4.2. Fruit fly – *Drosophila melanogaster*

Drosophila melanogaster (Figure 1.6), also known as the common fruit fly, is distributed worldwide and commonly found in close association with human habitation (Keller, 2007). Their cosmopolitan distribution was a relatively recent event, dated approximately in the early 19th century, and is largely attributed to human activity (Keller, 2007). Not much longer after this species had been introduced in the United States, following C. C. Woodworth's suggestion that *Drosophila* would be useful for genetic studies, W. E. Castle and T. H. Morgan set the first pioneering *Drosophila* genetics laboratories in Columbia and Harvard Universities respectively (Keller, 2007). Since then, mainly because *Drosophila* is a relatively easy and cheap species to maintain in laboratory, breeds quickly and lays many eggs, it has become a model organism not only for studies in the field of genetics, but also in physiology, pathology, biochemistry, developmental biology and evolution (Sang, 2001).

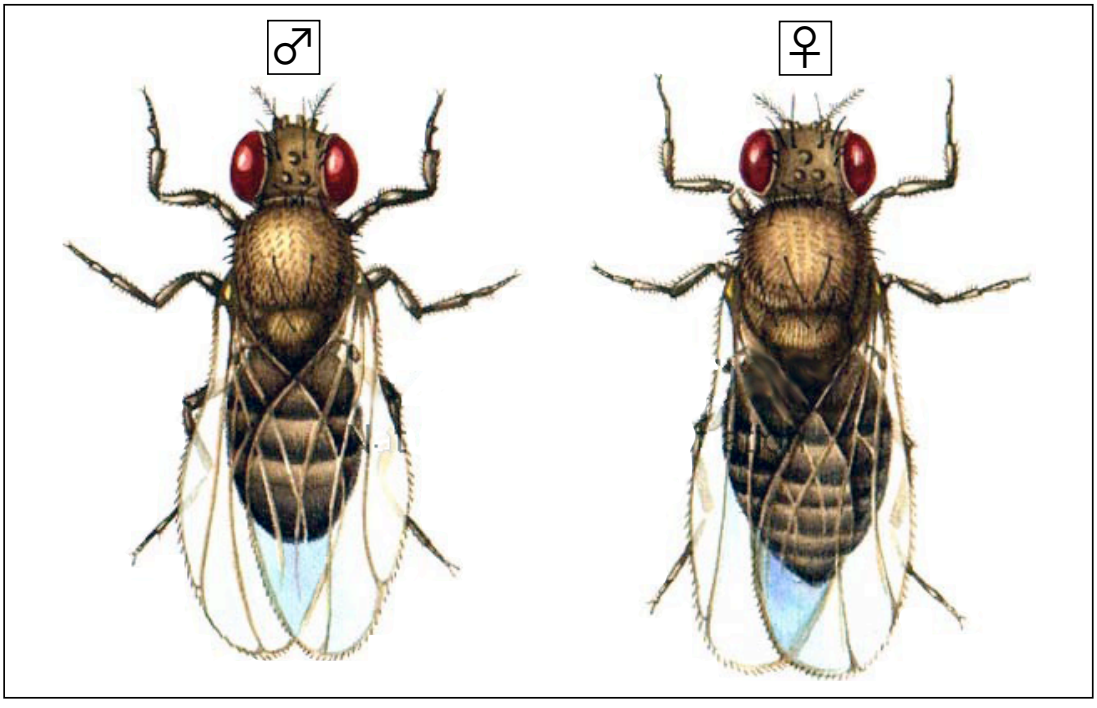

Figure 1.6. Male and female *Drosophila melanogaster*, or common fruit fly. Phylum: Arthropoda; Order: Diptera. First described by the German entomologist Johann Wilhelm Meigen in 1830. Watercolour illustration by Lizzie Harper, UK.

The period of development of a fruit fly from egg to adult varies with temperature as with most ectothermic species, but in average, this can be achieved within 7 days at 28 ºC (Ashburner and Novitski, 1976). Their life span is approximately 30 days at 29 ºC, and females become receptive to males within 8 to 12 hours after emergence. Larva hatch from laid eggs usually within 12-15 hours after fertilization, grow for about 4 days, and finally encapsulate into a puparium leading to another 4-day long metamorphosis from which an adult fly emerges (Ashburner and Novitski, 1976).

Drosophila oogenesis is divided in different stages (Bastock and St Johnston, 2008) (Figure 1.7), and germ line-associated mitochondria origin and segregation throughout these stages has been well characterised (Cox and Spradling, 2003). Initially, mitochondria is equally divided in the germ line stem cell and cystocyte divisions (Figure 1.7a, Region 1), subsequently, a portion of cystocyte mitochondria are funnelled into the oocyte via ring canals forming a large mass known as Balbiani bodies (Cox and Spradling, 2003). Balbiani bodies have been also detected in the early oocyte cytoplasm of various other species, which have led to the idea that they play a role in the development of germplasm (Matova and Cooley, 2001). Furthermore, Balbiani body mitochondria immunolablelling experiments using antibodies against mitochondrial ETC protein subunits were shown to be troublesome as weak or no signal could be detected, suggesting that the mitochondria entering the oocyte in the Balbiani body may have distinctive properties (Cox and Spradling, 2003). During oogenesis, cytoplasmic connections denominated "ring canals" connect the developing oocyte to nurse cells, from which mitochondria and other organelles move from the nurse cells to populate the oocyte (Cox and Spradling, 2003).

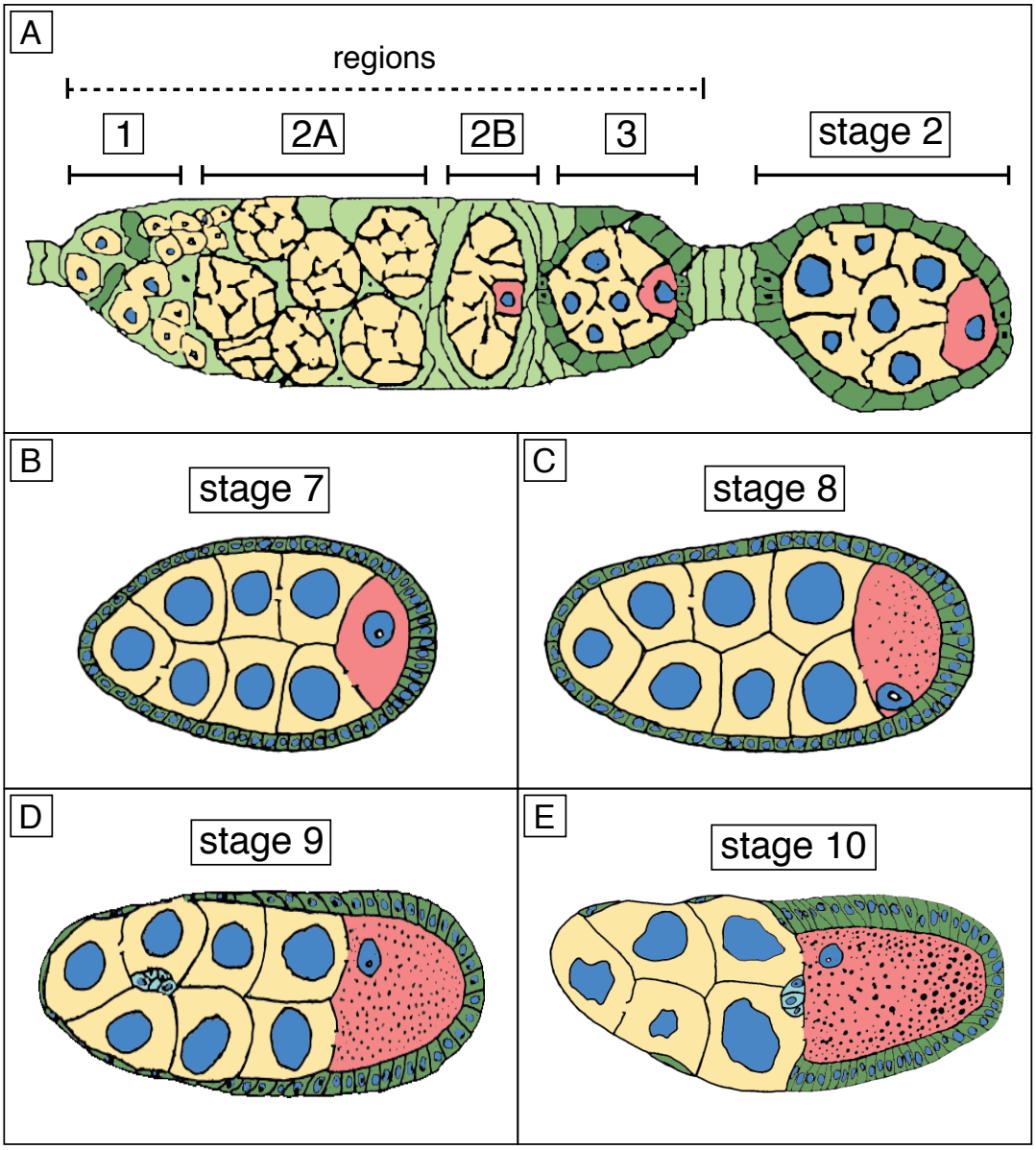

Figure 1.7. *Drosophila melanogaster* oogenesis. (a) The germarium is divided in regions 1, 2A, 2B and 3. Region 1 contains the germ line stem cells, region 2A and 2B are characterized by the presence of germ line cyst (yellow) made of 16 cells and the appearance of ring canals between them, and region 3 is the site of follicle cell (green) development. (b) Oocyte (red) repolarization occurs in stage 7, followed by (c) stage 9 and (d) stage 10 where follicle cells rearrange to contact the oocyte and the border cells migrate between the germ line nurse cells (yellow) towards the oocyte.

1.4.3. Zebrafish – *Danio rerio*

Zebrafish is a popular aquarium tropical fish, native of the southeastern region of the Himalayas (Mayden, et al., 2007). Nowadays, zebrafish is a common and useful model organism for studies of vertebrate development and gene function. The scientific importance of the zebrafish has been established by successful large-scale forward genetic screens, commonly referred to as the Tübingen/Boston screens (Mayden, et al., 2007). As a model biological system, the zebrafish offers many advantages for scientists. Its two genomes, nuclear and mitochondrial, have been fully sequenced, possess a relatively rapid embryonic development (Figure 1.8), and well-characterized mutant strains are readily available (Howe, et al., 2013).

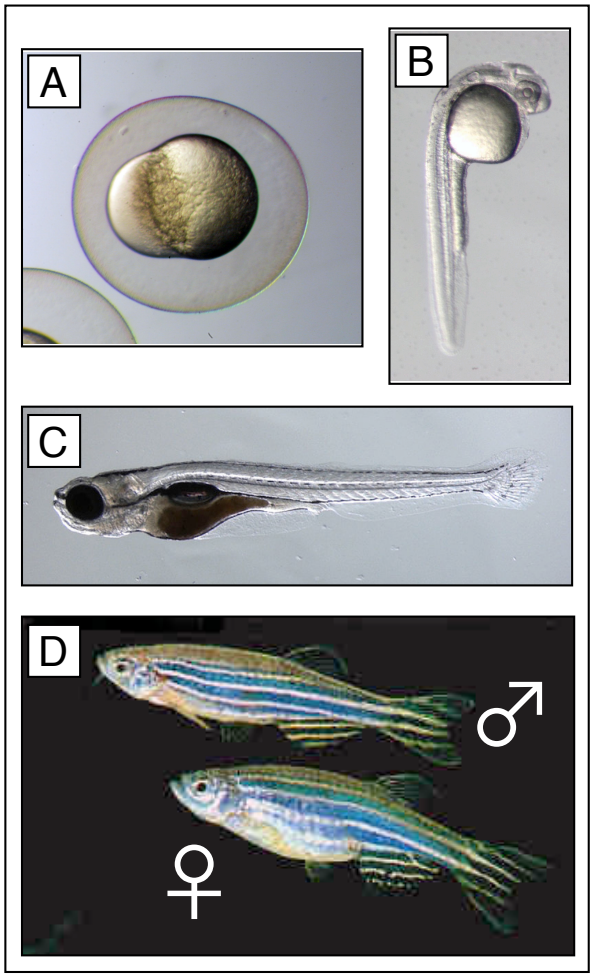

Figure 1.8. Different stages of zebrafish development. (a) a single-cell egg, (b) embryo, (c) larvae stage and (d) male and female adults. Images are copyright © 2002, Steve Bauskauf.

Zebrafish oogenesis can be divided in three main stages: primary growth, cortical alveolus stage and vitellogenic stage (Figure 1.9a) (Koç, et al., 2008). When the oocyte is mature, a layer of diploid follicle epithelium cells is seen as a thin layer of uniformly arranged nuclei (Figure 1.9b) (Koç, et al., 2008). Mitochondrial distribution in the zebrafish oocytes tends to follow a pattern also recognisable as mitochondrial clouds surrounding the nucleus (Zhang, et al., 2008), but not as organised as seen in *Drosophila* Balbiani bodies (Matova and Cooley, 2001).

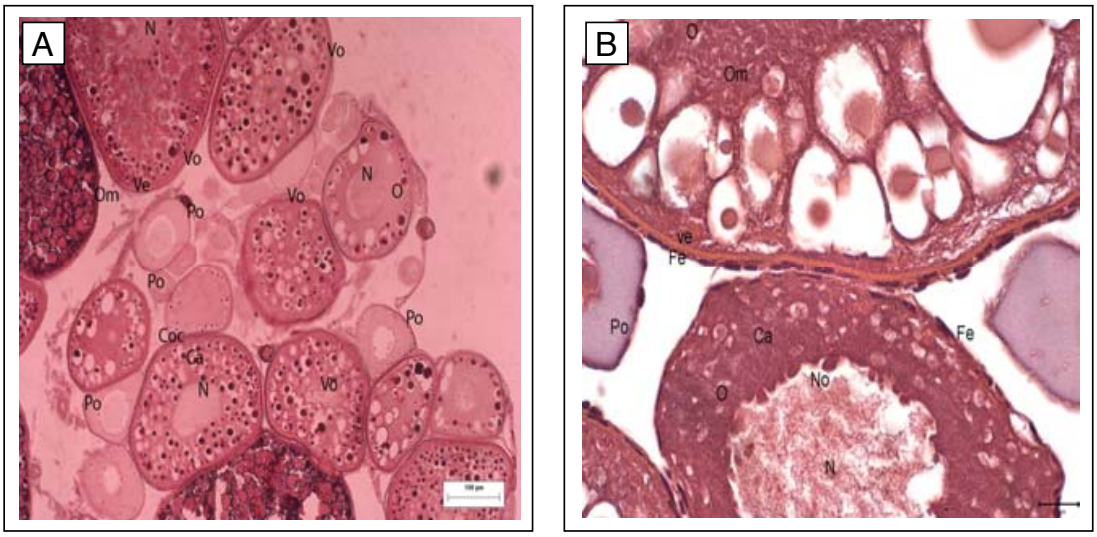

Figure 9. Zebrafish oogenesis stages. Images extracted from (Koç, et al., 2008).

The complete mitochondrial genome of zebrafish is approximately 16.6 kb in length. Its gene order and content resemble those of most vertebrates (Broughton, et al., 2001). The genome contains 13 protein-coding genes: 7 subunits of the NADH:ubiquinone oxidoreductase complex, one subunit of the ubiquinol:cytochrome c oxidoreductase complex, three subunits of the cytochrome c oxidase complex, and two subunits of ATP synthase (Broughton, et al., 2001). It also includes both small (12S) subunit and large (16S) subunit ribosomal RNA genes and 22 tRNA genes. A noncoding control region contains the origin of heavy strand replication, and the origin of light strand replication is found within a cluster of five tRNA genes (Broughton, et al., 2001).

1.5. Research Synopsis

The objective of my PhD research was to investigate the mitochondrial function in the oocyte of three highly diverged animals species, in the light of the predictions proposed by (Allen, 1996), as reviewed in section 1.3.5. The choice of the model species was based on their evolutionary divergence. *Aurelia aurita*, *Drosophila melanogaster* and *Danio rerio* have been diverged for at least 500 million years (Benton and Donoghue, 2006; Cartwright, et al., 2007). Nonetheless, all of them equally rely on anisogamy, uniparental inheritance, two separate sexes and the maintenance of a germ line for the continuity of their life cycles. What is the evolutionary force that has shaped and maintained such configuration in animals, for such a long time?

The proposed explanation is that a population of specialized cells, denominated the female germ line, are assigned to carry a special population of mitochondria that are quiescent for oxidative phosphorylation in order to protect its genetic material. To test this hypothesis, I used basic techniques in molecular biology, biochemistry and cell biology to investigate various aspects of mitochondrial bioenergetics of the female germ line in those three species, which includes mtDNA transcription, membrane potential, mitochondrial ultrastructure and reactive oxygen species production.

2

Materials and Methods

In 1930, the English evolutionary biologist and statistician R. A. Fisher, FRS wrote in the preface of his book The Genetical Theory of Natural Selection (Fisher, 1930):

"…what else should he do if he wishes to understand why the sexes are, in fact, always two? The ordinary mathematical procedure in dealing with any actual problem is, after abstracting what are believed to be the essential elements of the problem, to consider it as one of a system of possibilities infinitely wider than the actual, the essential relations of which may be apprehended by generalized reasoning, and subsumed in general formulae, which may be applied at will to any particular case considered."

2.1. Experimental systems

2.1.1. *Aurelia aurita*

Live male and female *Aurelia aurita* medusae were collected from Horsea Lake, Hampshire, UK on 28 August 2012 and 12 November 2012. Fresh tissue samples were taken from live specimens that had been maintained for up to 7 days in aerated artificial seawater diluted with distilled water to give a final salinity of 25, corresponding to the original brackish lake water.

2.1.2. *Drosophila melanogaster*

Drosophila melanogaster strains were maintained on standard cornmeal/yeast/agar medium at 25 °C. Wild type flies had been collected from Tannes, Italy. Bloomington stock #7194 P{sqh-EYFP-Mito} was used to visualize mitochondria (LaJeunesse, et al., 2004).

2.1.3. *Danio rerio*

Danio rerio wild-type strains (Tubingen and Tupfel long fin) were bred and raised in-house at the zebrafish facility of Queen Mary, University of London, U.K., as previously described by (Zimprich, et al., 1998). Work on zebrafish was conducted in accordance with the U.K. Animals (Scientific Procedures) Act 1986, with prior approval by the local institutional animal care committee. Adult fish were euthanized using a lethal dose of anesthetic (Tricaine methanesulfonate, MS-222) and tissues dissected immediately.

2.2. Mitochondrial gene expression analysis

2.2.1. Tissue dissection

Animals tissues were immersed in 1 X phosphate buffered saline (PBS) (Sigma #P4417) at room temperature and dissected using a stereomicroscope and extra sharp precision tissue forceps (VWR #232-0009). Dissected samples were placed in a 1.5 ml microcentrifuge tube and immediately stored in liquid nitrogen. Polypropylene pellet pestles (Sigma # Z359947) were used to grind the frozen samples to powder and RNA extraction was carried out immediately after.

2.2.2. RNA extraction

TRI Reagent® (Sigma # T9424) method was used to isolate total RNA. A volume of 500 µl of TRI Reagent® was added to the 1.5 ml microcentrifure tubes containing the frozen powder tissue samples. Gentle homogenization by inverting the tubes was done for 10 minutes at room temperature. 200 µl of cold chloroform was added and the solution was homogenized for 10 minutes at room temperature. Microcentrifuge tubes were centrifuged at 13,000 rpm for 30 min at 4 ºC, and 200 µl of the upper clear phase containing total RNA was transferred to a new microcentrifuge tube containing 500 µl of cold 100% ethanol and 100 µl of 7.5 M ammonium acetate (Sigma # A1542). Vortex was applied for 10 seconds and the solution was stored overnight at -20 ºC. Next day, microcentrifuge tubes were centrifuged at 13,000 rpm for 45 min at 4 ºC to pellet the RNA. Supernatant was discarded and cold 80% ethanol was added to wash the RNA pellet, mixed well with a vortex, and spun down at 13,000 rpm for 10 minutes at 4 ºC. This process was repeated once. After the second wash, the supernatant was carefully removed, and the RNA pellet was air dried for about 10 minutes at room temperature. Once the ethanol traces had completely evaporated, 50 µl of nuclease-free water (Life

technologies #AM9935) was used to dissolve the RNA pellet. RNA samples were further treated with DNase I (New England Biolabs #M0303) for 10 min at 37 ºC to ensure that there is no genomic DNA contamination, and re-purified using Pure LinkTM RNA Mini Kit (Life Technologies # 12183018A), all according to the manufacturer instructions. All samples were diluted to 5.0 ng/µl and stored at -80ºC.

2.2.3. RNA concentration measurement and quality analysis

RNA sample concentration was estimated using a NanoVue™ Plus Spectrophotometer (GE Healthcare #28-9569-66), according to the manufacturer's instructions. The instrument was blanked twice using 2.0 μl of nuclease-free water, and 2.0 μl of each sample was measure. A_{260}:A_{280} ratio was used to determine the relative amounts of protein contaminants (acceptable ratios ranged between 1.8 and 2.2), and A_{260}:A_{230} ratio was used to determine the relative amounts of other organic contaminants (acceptable ratios ranged between 1.8 and 2.2). To further analyse the RNA quality, samples were run in 1.5 % agarose for 30 minutes at 4 ºC. Sharp ribosomal RNA bands indicate the integrity of the RNA sample. Finally, all samples were diluted to 5.0 ng/µl concentration prior to the qPCR experiments.

2.2.4. Real-time qRT-PCR

Brilliant III Ultra-Fast SYBR® Green qRT-PCR (Agilent Technologies #600886) was used for the reactions, according to the manufacturer instructions. For a 20.0 µl reaction it was added 10.0 µl of 2X SYBR Green qRT-PCR master mix, 1.0 µl of 100 µM primer forward and reverse mix, 0.2 µl of 100mM DTT, 1.0 µl of RT/RNase, 1.0 µl of RNA template (5 ng/µl) and 6.8 µl of nuclease-free water. The primers used for the qRT-PCR reactions were designed using Primer3 (Rozen and Skaletsky, 2000) (Table 2.1). The reactions were placed in a Multiplate™ Low-Profile 96-Well Unskirted PCR Plates (Biorad #MLL-9651) and sealed with Optical Flat 8-Cap Strips (Biorad #TCS-0803). A Chromo4 Four-Colour Real-Time PCR system (Bio-Rad #CFB-3260G) and Opticon Monitor™ (v.3.1) software was used to run the reactions. Cycling program used is shown in Table 2.2.

Table 2.1. List of oligonucleotides used for the qRT-PCR experiments.

Gene		*A. aurita*	*D. melanogaster*	*D. rerio*
NADH: ubiquinone oxido-reductase subunit 1 (*nad1*)	forward primer	5'-GGA GTG GTT TGG GAC AGT CA-3'	5'-TTT GCT GAA GGA GAA TCA GAA TTA G-3'	5'-CGC TGG CAG AAA CAA ACC GAG C-3'
	reverse primer	5' -TCG AGG AAA GGG CCA AAT GT-3'	5'-AAT TAA AGC CAA ACC CCC TCT T-3'	5'-GGC AAA TGG TCC TGC TGC ATA CTC T-3'
	gene accesion	NCBI Ref. Seq.: NC_008446.1	FLYBASE ID: FBgn0013679	ZFIN ID: ZDB-GENE-011205-7
	amplicon size	102 bp	71bp	98 bp
Cytochrome bc_1 complex b subunit (*cob*)	forward primer	5'-AAC ATG GGC AGC ACC TAG TC-3'	5'-GAG GTG GAT TTG CTG TTG ATA ATG-3'	5'-TGC AAC CCT TAC ACG ATT CTT CGC A-3'
	reverse primer	5'-ACT TGG TTT CAG CTG TTC CCT-3'	5'-TCC TGT TTG ATG AAG GAA TAA TAA ATG-3'	5'-TGG GGT TTA GGC CAA GGG GGT-3'
	gene accesion	NCBI Ref. Seq.: NC_008446.1	FLYBASE ID: FBgn0013678	ZFIN ID: ZDB-GENE-011205-17
	amplicon size	149 bp	118 bp	125 bp
Cytochrome C oxidase subunit 1 (*cox1*)	forward primer	5'-ATG GTG GGA ACT GCC TTC AG-3'	5'-ATT GGT GGA TTT GGA AAT TGA TTA-3'	5'-CCC GAG CAT ACT TCA CAT CCG CC-3'
	reverse primer	5'-TTG ATC GTC CCC CAA CAT GG-3'	5'-GTT CCA GCT CCA TTT TCA ACT ATT-3'	5'-GCT CCT CCG TGG AGA GTG GCT-3'
	gene accesion	NCBI Ref. Seq.: NC_008446.1	FLYBASE ID: FBgn0013674	ZFIN ID: ZDB-GENE-011205-14
	amplicon size	75 bp	148 bp	91 bp

Table 2.1. List of primers used for the qRT-PCR experiments. (cont.)

Gene		*A. aurita*	*D. melanogaster*	*D. rerio*
***α*-tubulin**	forward primer	5'-CAG CAC CCC AAA TCT CCA CT-3'	–	–
	reverse primer	5'-ACC ATG AAA GCG CAG TCA GA-3'	–	–
	gene accesion	NCBI Ref. Seq.: AY226057.1	–	–
	amplicon size	94 bp	–	–
rpL32	forward primer	–	5'-CGA TAT GCT AAG CTG TCG CAC A-3'	–
	reverse primer	–	5'-CGC TTG TTC GAT CCG TAA CC-3'	–
	gene accesion	–	FLYBASE ID: FBtr0114555	–
	amplicon size	–	115 bp	–
***β*-actin**		–	–	5'-CGA GCA GGA GAT GGG AAC C-3'
		–	–	5'-CAA CGG AAA CGC TCA TTG C-3'
		–	–	ZFIN ID: ZDB-GENE-000329-1
		–	–	102 bp

Table 2.2 qRT-PCR cycling program

Cycles	Duration of cycle	Temperature	Step
1	10 minutes	50ºC	Reverse Transcriptase
1	3 minutes	95ºC	Initial Melting
40	5 seconds	95ºC	Melting
	10 seconds	60ºC	Annealing/Extension
35	2 seconds	55ºC to 90ºC*	Melting Curve

* with 1ºC increment per cycle.

2.2.5. Data Analysis

All messenger RNA quantities were normalized against nuclear encoded genes *α*-tubulin for *A. aurita*, *rpL32* for *D. melanogaster* and *β*-actin for *D. rerio*. A second normalization was done using intestine average values from both sexes as the calibration tissue. Three technical replicates for each biological replicate were averaged at the beginning, and three biological replicates were used to generate the error bars. C(t) values were analysed using qBase PLUS2 software (Biogazelle) (Hellemans, et al., 2007). One-way ANOVA with post-Tukey's multiple comparison test with a significance level set at $P \leq 0.05$ was used as statistical treatment.

2.3. Mitochondrial membrane potential imaging

2.3.1. Sample preparation

Live animal tissues were immersed in 1X PBS at room temperature and dissected using a stereomicroscope and extra sharp precision tissue forceps. Dissected samples were placed in a 1.5 ml microcentrifuge tube and Mitotracker® Red FM (Life Technologies # M22425) was added to the final concentration of 500 nM. For *A. aurita* and *D. rerio*, Mitotracker® Green FM (Life Technologies # M7514) was added simultaneously to the final concentration of 500 nM. Microcentrifuge tubes were placed in a rotator for 30 minutes at 30 rpm. DAPI (Life Technologies #D1306) was added to the final concentration of 1 μM when necessary. To wash the samples, the solution was carefully removed without disturbing the tissue sample, and 1.0 ml of fresh 1X PBS was added, followed by 5 minutes rotation at 30 rpm. This wash step was repeated twice. Samples were mounted in a drop of PBS on a W76×D26×H1 mm microscope glass slide (VWR # 631-1550); placed in between two flanking 15×15 mm microscope glass cover slips (VWR # 631-0655). A third cover slip was carefully placed on the top of the sample, but still supported by the a flanking cover slip in each side, ensuring that the samples were not squashed in between the slide and the cover slip. After mounted, the samples were immediately taken to the confocal microscope for image analysis.

2.3.2. Confocal light microscopy

Microscopy was performed using a Leica SP5 confocal microscope (Leica Microsystems). Excitation and emission wavelengths were selected for the different probes as follows: DAPI: 350 nm/450 to 480 nm, Mitotracker® Green FM: 488/500 nm to 530 nm, YFP: 514/520 nm to 560 nm, and Mitotracker® Red FM: 581/620 nm to 650 nm. Optimal excitation and emission wavelengths for each probe is shown in Figure 2.1. Imaging was performed with a 63 X lens, and laser output was kept under 15 % of maximal power. Leica LAS AF software was used to acquire and analyse the images.

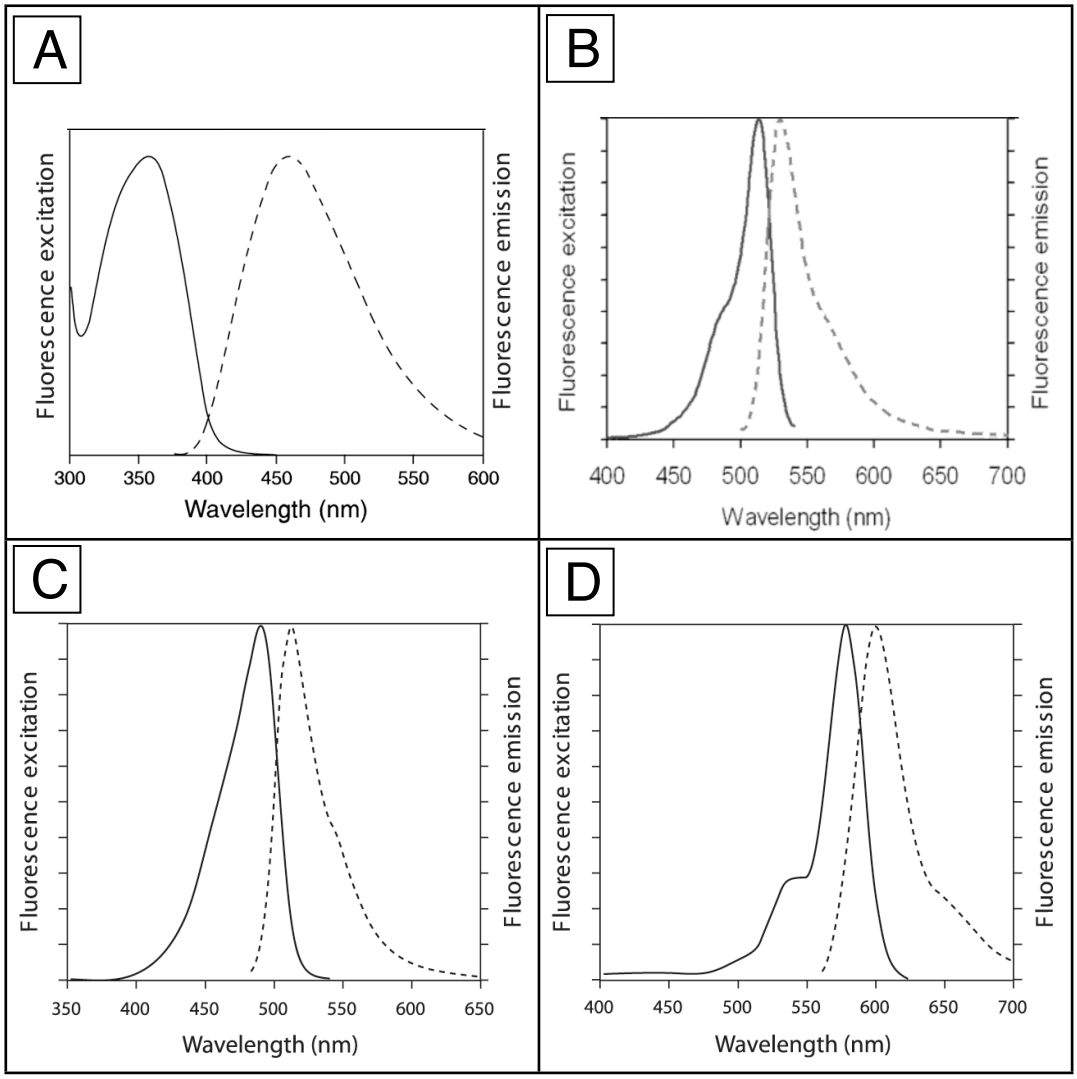

Figure 2.1. Range of excitation (solid line) and emission (dashed line) wavelengths for (a) 4',6-Diamidino-2-Phenylindole (DAPI), (b) Yellow-fluorescent protein (YFP) at pH 7.4, (c) Mitotracker® Green FM and (d) Mitotracker® Red FM. Images extracted from Life Technologies product manuals.

2.4. Reactive oxygen species (ROS) imaging

2.4.1. Sample preparation

Live animal tissues were immersed in 1X PBS at room temperature and dissected using a stereomicroscope and extra sharp precision tissue forceps. Dissected samples were placed in a 1.5 ml microcentrifuge tube and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Life Technologies #D-399) was added to the final concentration of 1 μM. Microcentrifuge tubes were placed in a rotator for 30 minutes at 30 rpm. To wash the H_2DCF-DA excess off the samples, the solution was carefully removed without disturbing the tissue sample, and 1.0 ml of fresh 1X PBS was added, followed by 5 minutes rotation at 30 rpm. This wash step was repeated twice. In the final wash, DAPI was added to the final concentration of 1 μM, and this solution was used to mount the samples on the microscope slides. Samples were mounted as described in Section 2.3.1. After mounted, the samples were immediately taken to the confocal microscope for image analysis.

2.4.2. Confocal light microscopy

Microscopy was performed using a Leica SP5 confocal microscope (Leica Microsystems). Excitation and emission wavelengths were selected for the different probes as follows: DAPI: 350 nm/450 to 480 nm and H_2DCF-DA: 488 nm/520-550 nm. Imaging was performed with a 63 X lens, and laser output was kept under 15 % of maximal power. Leica LAS AF software was used to acquire and analyse the images.

2.5. Transmission electron microscopy (TEM)

2.5.1. Sample preparation

For transmission electron microscopy (TEM), specimens were fixed overnight in 2.5 % (v:v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), except for the *Aurelia aurita* samples, which were fixed overnight in 2.5 % (v:v) glutaraldehyde in filter sterilized seawater to maintain the osmotic equilibrium with the samples. All samples were post-fixed in 1 % (w:v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 1.5 hours, and then dehydrated through a graded ethanol series, equilibrated with propylene oxide before infiltration with TAAB SPURR resin and polymerised at 70 °C for 24 hours. Infiltration times were increased to 48 hours for Drosophila samples, to aid resin penetration through the abdominal cuticle. Ultra thin sections (70-90 nm) were prepared using a Reichert-Jung Ultracut E ultramicrotome, mounted on 150 mesh copper grids, contrast-stained using uranyl acetate and lead citrate.

2.5.2. TEM imaging

Ultra thin sections were examined on a FEI Tecnai 12 transmission microscope operated at 120 kV. Images were acquired with an AMT 16000M digital camera.

2.5.3. Stereological analysis

Stereological analysis was done with the assistance of Dr. Gema Vizcay-Barrena (CUI, King's College, London) according to (Howard and Reed, 2010). Thirty mitochondria from each group were aligned on a combined point counting grid composed of two sets of points of different densities on the same grid; 9 fine

points per coarse point. The volume of reference (mitochondria) was estimated by counting the number of coarse points that hit the reference space, and multiplying by 9. The volume of the particular phase (cristae) was estimated by counting the number of fine and coarse points that hit the cristae. Statistical *t*-test analysis was carried out in Microsoft Excel, and the S.E.M. was used to generate the error bars. The significance level was set at $P \leq 0.01$.

3

Mitochondrial DNA transcription in the female germ line

In 1998, the British palaeontologist R. A. Fortey, FRS wrote in his book Life: an Unauthorized Biography (Fortey, 1998):

"Exactly how sexual differentiation originated is still hotly debated… It must be stated that it was evidently an ancient, Precambrian innovation because so many plants and animals – even fungi – show evidence of sexual reproduction. The sexual imperative runs into deep time."

3.1. Introduction

Most of what is known on the transcriptional regulation of oocytes concerns only nuclear genes, while only a few studies, so far, have attempted to investigate how mitochondrial transcription behaves in those cells. Nevertheless, recent studies aiming to characterise transcriptional factors may have elucidated potential pathways for regulation of mitochondrial transcription, including the ones specific to the oocytes and early stages of embryonic development.

It has been known that the nuclear transcriptional factor *Oct-4* is selectively expressed in totipotent embryonic stem and germ cells of most animals (Song and Wessel, 2005). The set of genes regulated by *Oct-4*, and other oocyte-specific nuclear transcriptional factors have been identified by a comprehensive microarray study of the human oocyte transcriptome (Kocabas, et al., 2006). The classes of nuclear-encoded genes found to be highly up-regulated in the human oocyte belong mainly to RNA, DNA, and protein metabolism, as well as genes involved in chromatin modification (Kocabas, et al., 2006). Despite of the large amount of data analysed, the word "mitochondria" was not mentioned a single time in their study. Unfortunately, a great deal of information on mitochondrial function acquired by such high throughput studies is often neglected and permanently lost (Pesole, et al., 2012).

Despite the bacterial origins of mitochondria, their original bacterial-type transcription machinery has been replaced in the course of evolution, and now relies solely on a bacteriophage-type RNA polymerase (RNAP) to transcribe their genes (Greenleaf, et al., 1986). The only known exceptions, so far, are the species of the protist order Jakobid, which retain a mitochondrial-encoded bacterial RNAP and its sigma factors (Lang, et al., 1997). In contrast to bacteriophage RNAPs, mitochondrial RNAPs are not fully functional as single subunits, and require transcription factors to effectively transcribe the mtDNA (Shoubridge, 2002).

Mitochondrial transcription factor 1 (Mtf1), from *Saccharomyces cerevisiae*, was the first mitochondrial RNAP transcription factor to be characterised. It resembles a bacterial sigma factor and is required for transcriptional specificity in yeast (Jang and Jaehning, 1991). Two homologues of the *S. cerevisiae* Mtf1, named mitochondrial transcription factor B1 and B2 (TFBM1 and TFBM2 respectively), are also required for efficient mitochondrial transcription in mammals (Falkenberg, et al., 2002). In addition, mitochondrial RNAP function was shown to be drastically impaired in the absence of the mitochondrial transcriptional factor A (TFAM) (Larsson, et al., 1998). Another class of mitochondrial transcription factors recently characterised is the transcription elongation factor of mitochondria (TEFM), found to interact with and enhance *in vitro* activity of the human mitochondrial RNAP (Minczuk, et al., 2011).

Earlier studies in mice have provided evidence that mitochondrial transcription remains inactive in the oocyte and 2-cell embryo, but it is reinitiated and undergoes a 30-fold rise during cleavage through the blastocyst stage (Piko and Taylor, 1987; Taylor and Piko, 1995). More recent findings have shown that not only the mitochondrial RNAP nuclear gene expression is up-regulated during cleavage stages (Kameyama, et al., 2007), but also the mitochondrial-encoded respiratory subunits *nad4* (Kameyama, et al., 2007) and *cox1* (May-Panloup, et al., 2005). In porcine oocytes, TFAM mRNA quantities also increase significantly from the germinal vesicle (GV) stage up to the blastocyst stage (Antelman, et al., 2008). Furthermore, TFAM protein concentrations were found to be 3-fold higher in artificially activated parthenogenic oocytes compared to GV and metaphase II (MII) stage oocytes (Antelman, et al., 2008).

There is some evidence that mitochondrial transcription is suppressed in oocytes and early stages of embryonic development, but so far this has been shown only in mammals. If there is such a fundamental mechanism to suppress the bioenergetic activity of oocyte mitochondria via transcription suppression as proposed by (Allen, 1996), then it should be seen in different branches of the animal kingdom, and also for other respiratory genes.

In this study, I present results on the gene expression of key subunits of the respiratory ETC encoded by the mitochondrial genome (Figure 3.1) of *Drosophila*, zebrafish and jellyfish ovaries, relative to male gametic tissue and other somatic tissues, in reference to a housekeeping nuclear gene. Results for the plant species *Arabidopsis thaliana*, *Nicotiana tabacum* and *Fucus vesiculosus* are also presented in this chapter.

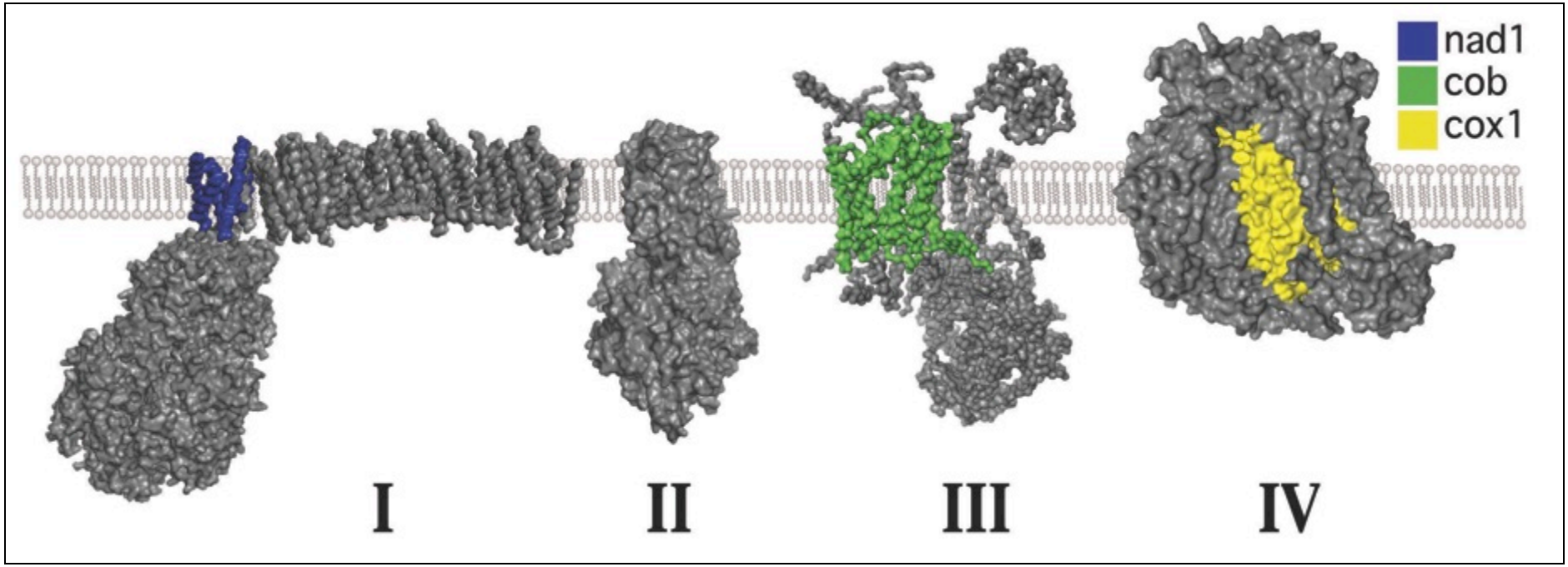

Figure 3.1. A schematic representation of the respiratory electron transport chain of the mitochondrial inner membrane. The protein subunits highlighted in blue, green and yellow represent the products of the mitochondrial genes *nad1*, *cob* and *cox1* respectively. Structures are surface models drawn using PyMol (Schrodinger, 2010) from PDB atomic coordinate files with the following accession numbers: Respiratory Complex I (NADH-ubiquinone oxidoreductase), 3M9S; Respiratory Complex II (succinate dehydrogenase), 1ZOY; Respiratory Complex III (the cytochrome b-c1 complex), 1QCR; Complex IV (cytochrome c oxidase), 1V54.

3.2. Results

3.2.1. Mitochondrial transcription is suppressed in jellyfish ovary

To estimate the relative quantity of mitochondrial transcript of one polypeptide subunit from each of the three proton-motive respiratory chain complexes outlined schematically in Figure 3.1, real-time qRT-PCR analysis was performed. Figure 3.2 shows the relative quantity of mRNA from the genes *nad1*, *cob* and *cox1* in somatic tissue samples from the bell, oral arm, and gonads of male and female *Aurelia aurita* medusae, which is the motile reproductive phase of jellyfish. For ovary and testis samples, a mixed population of gamete cells and surrounding diploid cells were pooled together. For all three genes, mRNA is lowest in ovary, while testis mRNA quantities are close to those of somatic tissue, notably the male bell. In males, bell, with a high proportion of contractile tissue, appears to have the most mRNA for all three mitochondrial genes, and especially for the *cox1* subunit of cytochrome oxidase, the terminal electron acceptor for aerobic respiration. Similarly, testis mitochondria seem to have higher amounts of *cox1* transcripts. This result fits with the assumption that oxygen supply may be a limiting factor for contractions of the bell and sperm flagellar movement. Bell contraction provides propulsion and, together with osmoconformation (Hirst and Lucas, 1998), maintains position in the water column.

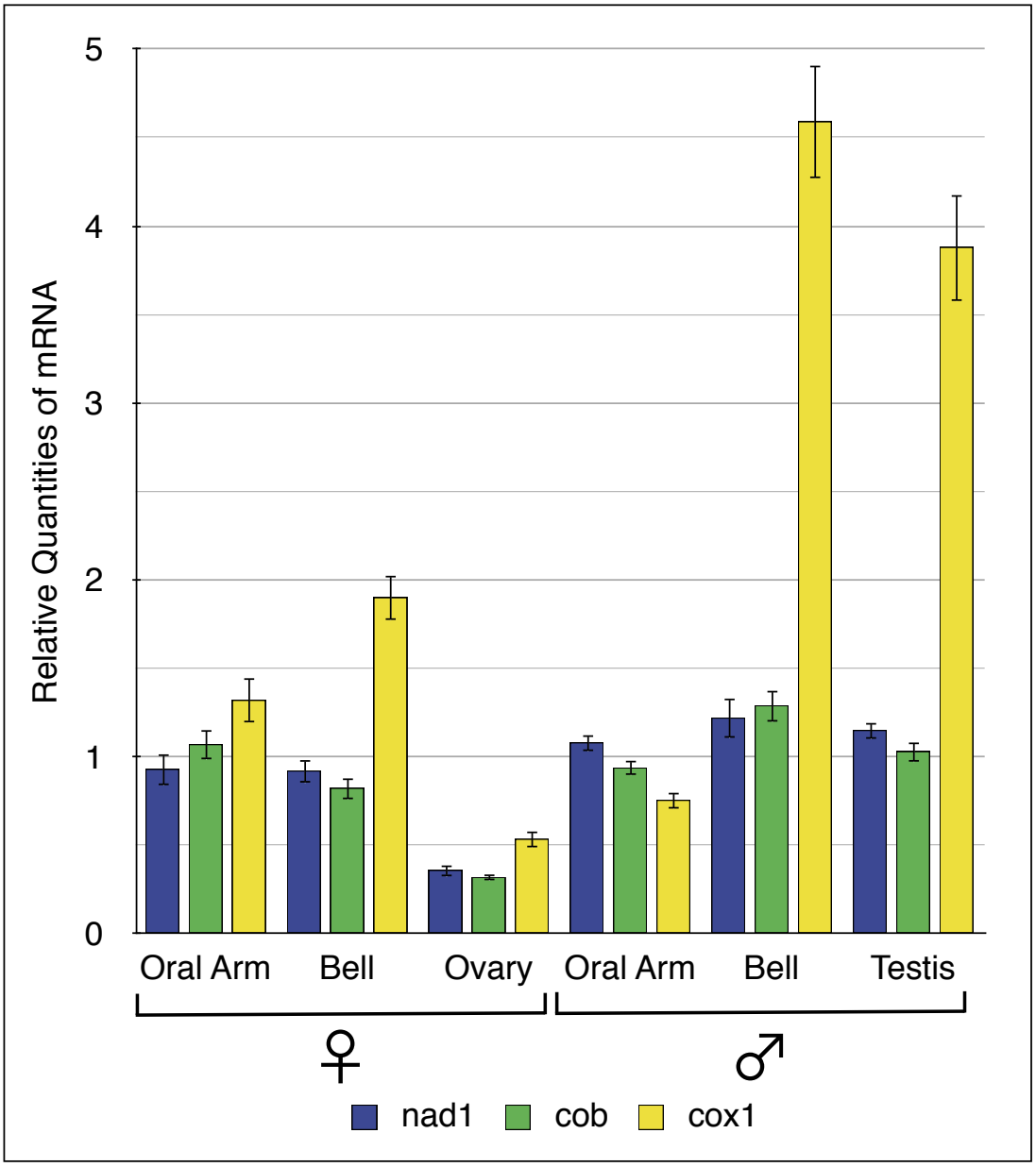

Figure 3.2. Relative quantities of mRNA from the mitochondrial genes *nad1* (blue), *cob* (green) and *cox1* (yellow) in different tissue samples from male and female medusae of the jellyfish, *Aurelia aurita*. mRNA quantities are colour coded according to the gene transcribed, as indicated also in Figure 3.1. Mitochondrial mRNA quantities are expressed relative to those from the nuclear gene α-tubulin, and the ratio then expressed relative to the corresponding value for oral arm somatic tissue. Error bars stand for +s.e.m. of three biological replicates. $P \leq 0.05$.

3.2.2. Mitochondrial transcription is suppressed in *Drosophila* ovary

The same gene expression analysis was performed in *Drosophila melanogaster* tissues (Figure 3.3). It is seen that the mRNAs for the three mitochondrially-encoded genes are present in similar relative quantities in somatic tissues, intestine and thorax, from both male and female *Drosophila*. Thorax tissue consists predominantly of insect flight muscle, which has a high volume-specific demand for oxidative phosphorylation, consistent with it also having the highest observed relative values for mRNA transcribed from mitochondrial genes for the three respiratory chain proteins. Intestine, a less energetically demanding somatic tissue, has lower relative mitochondrial mRNA quantities from all three mitochondrial genes, and in both sexes. For these somatic tissues, there is little difference in respiratory chain mRNA between males and females, with the possible exception of thorax *cob* and, to a lesser extent, *cox1*, both of which appear to have slightly more RNA in males. In sharp contrast with somatic tissue, ovaries and testis exhibit a marked sex-specific difference for all three respiratory chain mRNA quantities for *nad1*, *cob* and *cox1*. Quantities of the gene transcripts in *Drosophila* ovary are approximately 15-fold lower than flight muscle, and 3.2-fold lower than intestine.

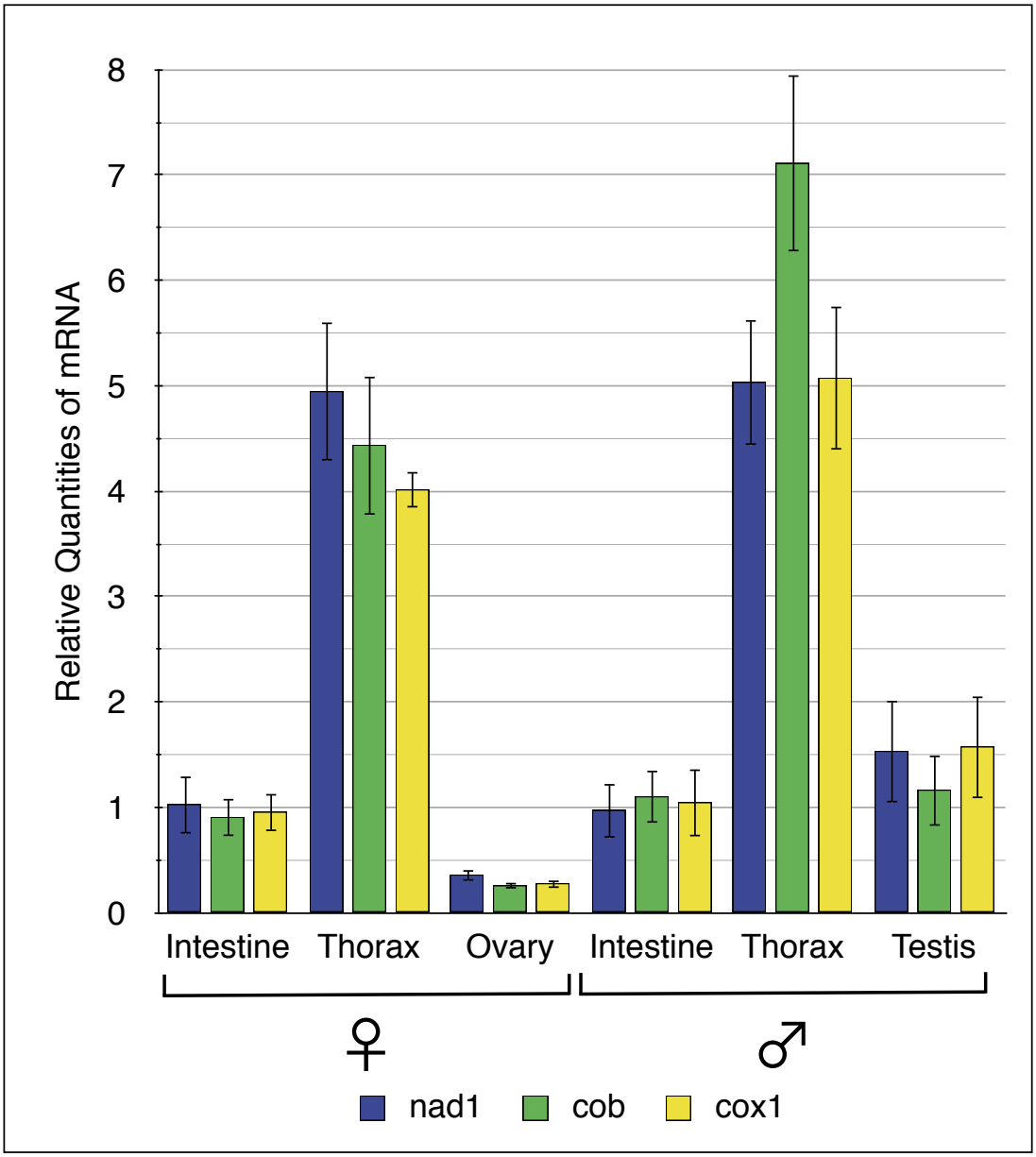

Figure 3.3. Relative quantities of mRNA from the mitochondrial genes *nad1* (blue), *cob* (green) and *cox1* (yellow) in different tissue samples from male and female fruit fly, *Drosophila melanogaster*. mRNA quantities are colour coded according to the gene transcribed, as indicated also in Figure 3.1. Mitochondrial mRNA quantities are expressed relative to those from the nuclear gene *rpL32*, and the ratio then expressed relative to the corresponding value for intestine somatic tissue. Error bars stand for +s.e.m. of three biological replicates. $P \leq 0.05$.

3.2.3. Mitochondrial transcription is suppressed in zebrafish ovary

Mitochondrial mRNA quantities from somatic tissue and from male and female gonads of zebrafish, *Danio rerio*, is shown in Figure 3.4. Consistent with the previous two species, swimming skeletal muscle shows the highest mRNA quantities, consistent with its mechanochemical energetic requirement and thus demand for ATP from mitochondrial oxidative phosphorylation. Differences are seen between the mRNAs for the three genes in female muscle, but not in male. The most marked difference between the sexes is, again, in gonads, with the smallest quantities of all three mRNAs being recorded for ovary. In summary, transcription of mitochondrial genes for respiratory chain subunits is lowest in ovaries, approximately 15-fold lower than skeletal muscle, and 5.5-fold lower than intestine.

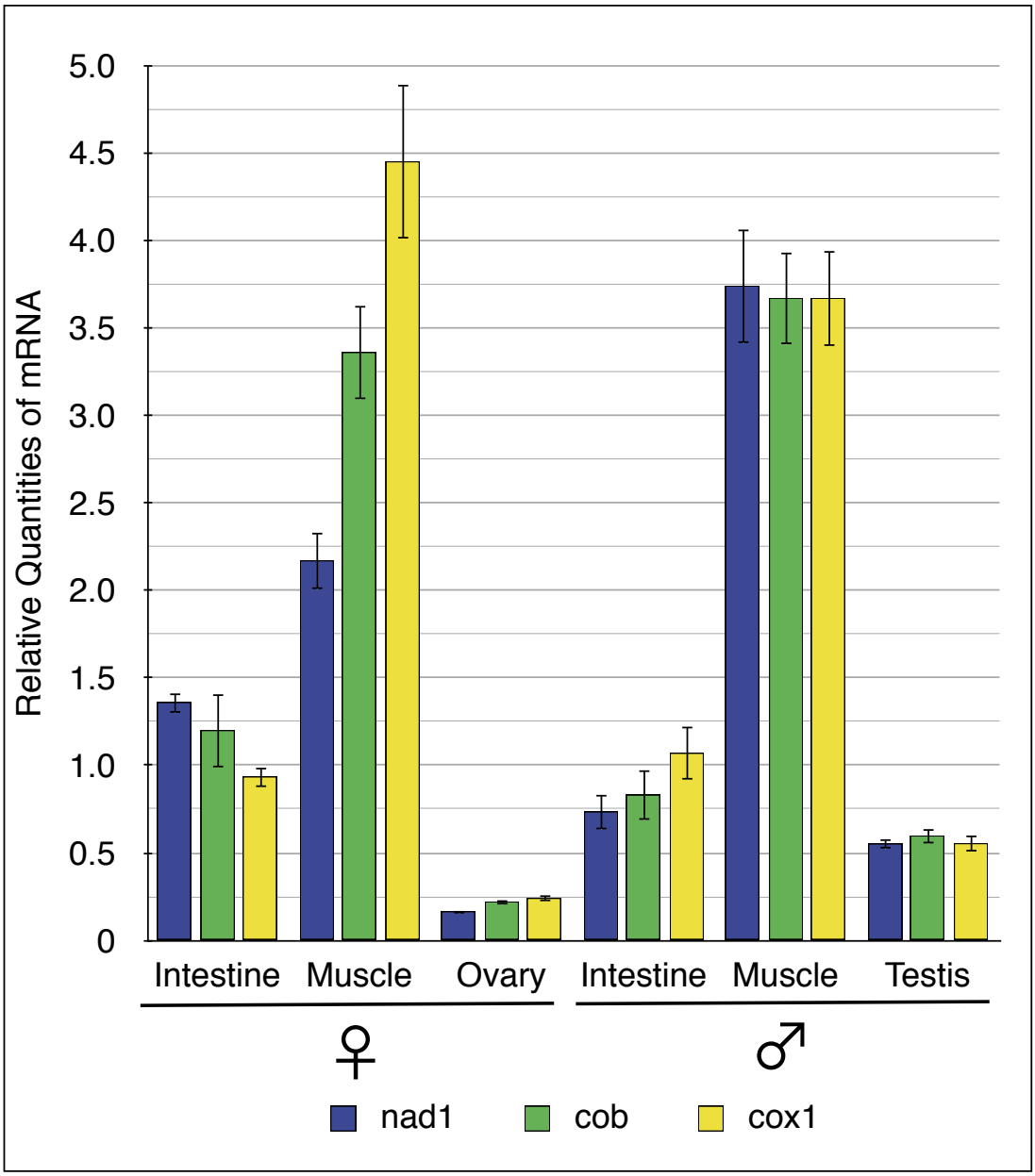

Figure 3.4. Respiratory electron transport gene expression profile of the zebrafish, *Danio rerio*. Quantities are shown for mitochondrial mRNA expressed in different tissues of male and female individuals. The colour coding is the same as that used in Figure 3.1. Blue, *nad1*; green, *cob*; yellow, *cox1*. Error bars indicate S.E.M. $P \leq 0.05$.

3.2.4. Is there sex-specific mitochondrial transcription in plants?

To check whether plants also exhibit the same sex-specific mitochondrial transcription pattern seen in animals, qRT-PCR was carried out to detect the quantities of *cox1* expressed in different tissues of two monoecious land plant species: *Nicotiana tabacum*, *Arabidopsis thaliana*, and a dioecious brown algae species *Fucus vesiculosus*.

In land plants (Figure 3.5), the amount of *cox1* transcripts relative to the nuclear encoded housekeeping gene *actin8* was found to be the lowest in the petals. In contrast to the animals species studies (Figures 3.2-3.4), tobacco ovary exhibited the same levels of *cox1* expression as seen for active somatic leaf tissue, while even higher levels of *cox1* expression were detected in *Arabidopsis* ovary. Male gametic tissue, or anthers, showed the highest levels of *cox1* expression in both species.

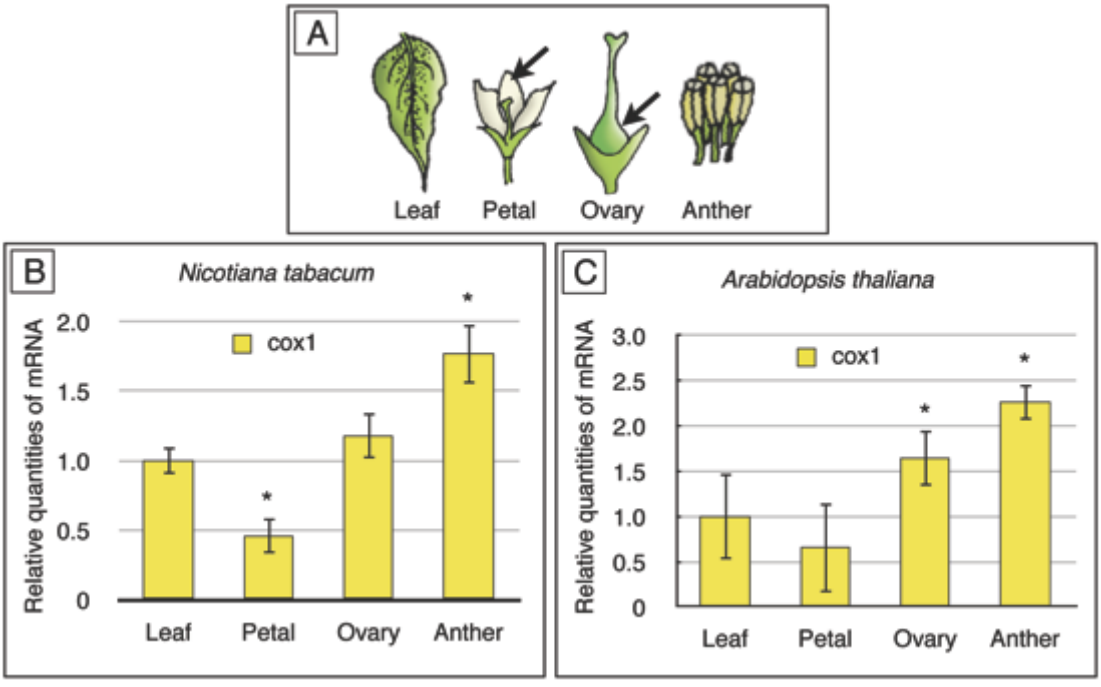

Figure 3.5. Gene expression levels of the mitochondrial-encoded cytochrome oxidase subunit 1 (*cox1*) in different plant tissues. (a) Plant organs from which the samples were dissected for the analysis. Relative quantities of *cox1* transcripts for (b) *Nicotiana tabacum*, and (c) *Arabidopsis thaliana*. (*) indicates P≤0.05 in comparison with leaf. Error bars were generated by S.E.M.

Similarly to the land plants, the brown algae *Fucus vesiculosus* (Figure 3.6) seemed not to suppress mitochondrial transcription in the female gametes. Relative quantities of *cox1* transcripts in relation to the nuclear encoded *β*-actin gene were measured for lamina, shoot, sperm and eggs. Both female and male gametes exhibited similar *cox1* expression levels as photosynthetically active shoot, and lamina tissue. However, interestingly, sterile filaments, which are the precursor cells for the gametes in male and female *Fucus*, were found to have lower amounts of *cox1* transcripts that what is seen for shoot and male lamina. Moreover, female lamina showed a three-fold higher *cox1* expression level compared to the male lamina samples; however, this difference may be due to the quality and/or age of the samples collected from the sea.

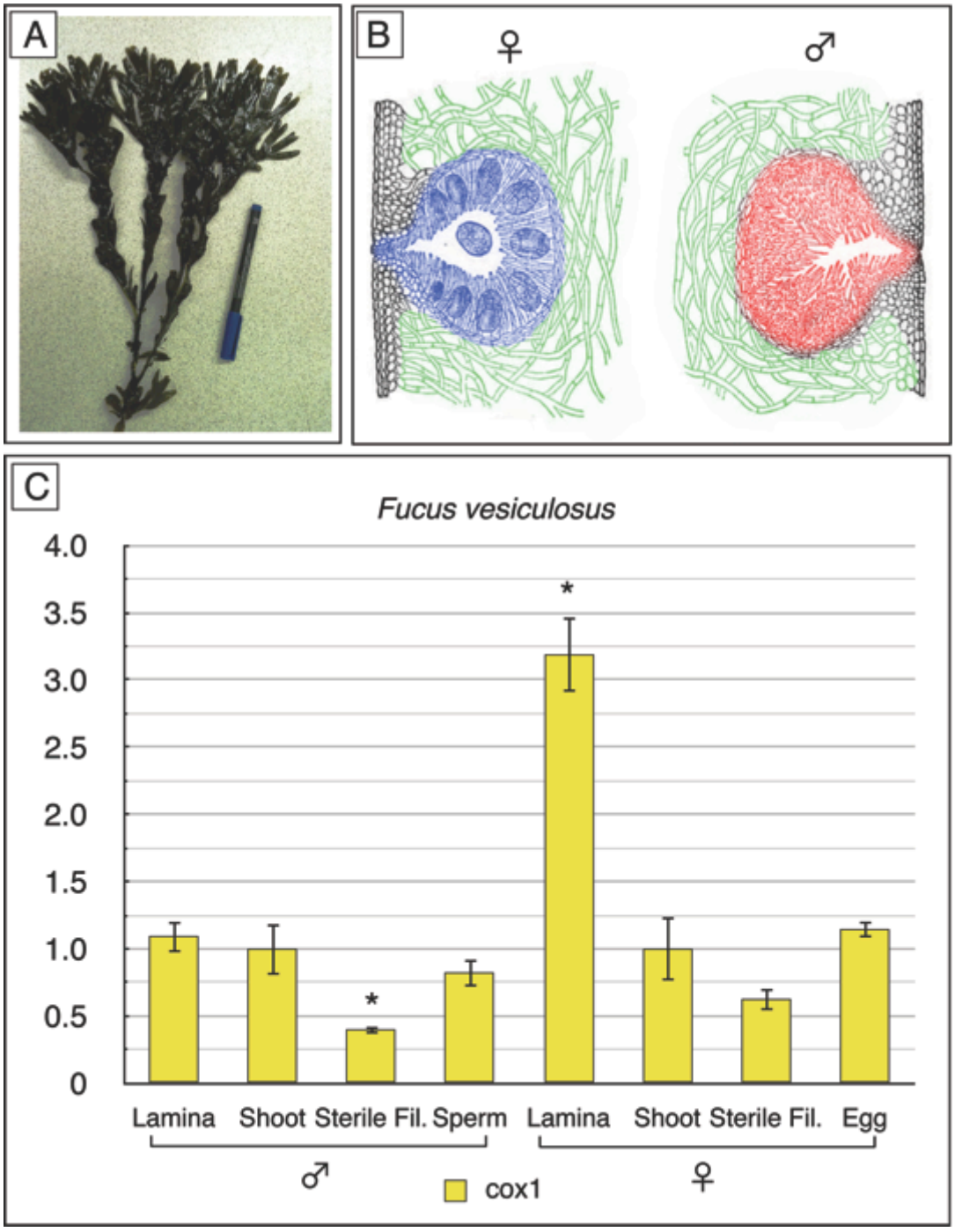

Figure 3.6. *Cox1* expression in *Fucus*. (a) Photograph of a female *Fucus vesiculosus* at early reproductive stage, with a pen as scale object, (b) conceptacles showing tissues used in the analysis: sterile filament in green, eggs in blue and sperm in red. Image modified from (Gray, 1887). (c) *Cox1* expression levels. (*) indicates P≤0.05 in comparison with shoot.

3.3. Discussion

The results presented in this chapter provide evidence that mitochondrial transcription is suppressed in the ovaries of jellyfish, fruit fly and zebrafish. MtDNA transcriptional rate has been correlated with energy output (Mehrabian, et al., 2005; Virbasius and Scarpulla, 1994). Therefore, decreased amount of transcripts for three mitochondrial genes, which encode subunits of respiratory electron transport chain complexes, may suggest that female germ line mitochondria are indeed bioenenergetically quiescent, as hypothesised before by (Allen, 1996). Previous pre-implantation studies in mice have reported similar results. It has been shown that oocyte and 2-cell stages have reduced mRNA quantities for the mitochondrial genes 12 and 16 S rRNAs, *cox1* and *cox2* (Piko and Taylor, 1987), *cox4*, *cox5b*, *cox7* and *atp9* (Taylor and Piko, 1995), and *nad2* and *nad6* (Thundathil, et al., 2005). Mitochondrial transcription is suggested to become active only from cleavage stages onwards (Piko and Taylor, 1987), when mitochondrial transcription factors start to be transcribed from the nuclear genome (Antelman, et al., 2008; Spikings, et al., 2007).

Other proteins could also be involved in regulating transcription in early stages of female germ line development. Mitochondrial single-stranded DNA binding protein (mtSSB) has been shown to be abundant in mitochondria of primary and mature oocytes of *Xenopus laevis* (Ghrir, et al., 1991), *Drosophila melanogaster* (Maier, et al., 2001) and *Caenorhabditis elegans* (Sugimoto, et al., 2008). Interestingly, mtSSB seems to have a dual function in animal mitochondria: at the same time that it promotes mtDNA replication by enhancing mitochondrial DNA polymerase activity (Farr, et al., 2004; Farr, et al., 1999), it also inhibits transcription of the mtDNA (Barat-Gueride, et al., 1989). Furthermore, binding activity of mtSSB to the mtDNA appears to be regulated by phosphorylation mediated by Cyclin-dependent kinase (CDK) (Radulovic, et al., 2010), suggesting that the control of cell division might be linked with mitochondrial activity.

Plants do not possess a dedicated female germ line. This may explain why no difference in mitochondrial transcript levels was found between female and male gametes, and also between female gametes and somatic tissue (Figures 3.4 and 3.5). In land plants, the actual female and male gamete cells, denominated megasporocyte and microsporocyte, respectively, are surrounded by many layers of modified leaves, which raises the possibility that the actual gamete cell is a very small proportion of the total ovarian tissue used in this analysis. This problem could also be solved by single cell qPCR techiniques (Taniguchi, et al., 2009). However, if there was any sex-specific difference in the mitochondrial transcription, in should have been detected in the dioecious brown algae *Fucus*, which contains a much larger proportion of eggs and sperms in their conceptacles compared to surrounding somatic tissues. Another particularity from plants is the chloroplast, which contains DNA and is also inherited from the cytoplasm, but not always only from the maternal side. Paternal inheritance of chloroplast DNA has been previously shown for the genuses *Larrea* (Yang, et al., 2000), *Pinus* (Chen, et al., 2002), *Actinidia* (Cipriani, et al., 1995), *Passiflora* (Hansen, et al., 2007), *Helianthus* (Ellis, et al., 2008), among others. If the assumption that organellar DNA must be free from ROS to ensure accurate transmission between generations (Allen, 1996) also applies to plants, then plants must have developed more specialised mechanisms to ensure the maintenance of cytoplasmic genomes, without necessarily relying on maternal inheritance and a protected germ line to do so.

One caveat of this set of experiments is that, for ovary and testis samples, a mixed population of gametic and somatic cells were pooled together for the gene expression analysis – for example, ovary samples contained not only gametes, but also their surrounding diploid follicle cells. Nonetheless, *Drosophila* and zebrafish ovary and testis samples are mostly comprised of haploid gametes; therefore, for this study, it is reasonable to consider that the results seen for those species represent mostly the mitochondrial transcription of gamete cells as opposed to somatic diploid cells. In the case of jellyfish, the male and female gonads contain a higher proportion of somatic tissue, which may explain why less difference was detected in those samples, compared to the somatic tissues in the other two animal species. Recent techniques

of quantitative analysis of gene expression in a single cell by qPCR (Taniguchi, et al., 2009) could allow a more specific assessment of mitochondrial transcription in a single-cell egg, or even in isolated sperms. Furthermore, in combination with single-cell qPCR, high-throughput transcriptome analysis by RNA-sequencing (Wang, et al., 2009) would enable a global survey of the expression of all mitochondrial genes, and give a much more accurate estimate of the amounts of mitochondrial transcripts present in each cell type.

4

Mitochondrial membrane potential in the female germ line

In 1978, the British biochemist Peter D. Mitchell, FRS said during his Nobel Prize award speech (Mitchell, 1978):

"The final outcome cannot be known, either to the originator of a new theory, or to his colleagues and critics, who are bent on falsifying it. Thus, the scientific innovator may feel all the more lonely and uncertain."

4.1. Introduction

Mitochondrial respiratory electron transport chain (ETC) proteins oxidise substrates produced in the citric-acid cycle to generate a proton gradient across the inner membrane, thus generating two components: a membrane potential, and a pH gradient (Mitchell, 1979). These two components store energy that is ultimately used to drive the conversion of ADP and Pi into ATP by the F_0F_1-ATP synthase complex via a chemiosmotic mechanism (Mitchell, 1961). Chemiosmotic ATP synthesis is undoubtedly the most ancient biological process, and thought to have been present in the last universal common ancestor of cells (LUCA) (Lane, et al., 2010). Because of such high degree of conservation, it is hypothesised that, initially, geological chemiosmosis provided the thermodynamic force for origin of life, and eventually LUCA achieved independence from the geological source by acquiring the ability of synthesising their own lipidic membrane and chemiosmotic machinery (Martin and Russell, 2003; Martin and Russell, 2007).

The chemiosmotic electrochemical gradient generated by proton pumping is expressed as a membrane potential ($\Delta\Psi_m$) of approximately -180 mV, and to a smaller extent a pH gradient (ΔpH_m) equivalent to about -60 mV, yielding a total of -240 mV of energy (Mitchell, et al., 1981). Monitoring membrane potential using fluorescence probes and light microscopy has been extensively used to estimate mitochondrial $\Delta\Psi$ in a single-cell (Johnson, et al., 1981; Perry, et al., 2011). Those fluorescent probes are generally lipophilic compounds with a positive charge delocalised throughout the whole molecule, and equilibrate according to the Nernst potential (Figure 4.1.) accumulating in the mitochondrial matrix in inverse proportion to $\Delta\Psi_m$ (Chen, 1988). Rhodamine 123 (Figure 4.2a) was one of the first lipophilic fluorescent probes successfully used to measure mitochondrial membrane potential (Chen, 1988), and since then other probes have been further developed and become commercially available.

The combination of a fluorescent mitochondrial marker (i.e. transgenic organism containing a mitochondrial targeted-YFP, or Mitotracker Green FM),

which reports the presence of mitochondria independently of their $\Delta\Psi$, with a $\Delta\Psi_m$-dependent lipophilic fluorescent probe (i.e. Mitotracker Red, TMRM) has been proven to be a powerful tool to estimate the $\Delta\Psi_m$ of a single mitochondrion in live tissue or in isolated samples (Schwarzlander, et al., 2012). Another reliable method to measure mitochondrial membrane potential and proton leak uses a combination of a TPP^+ ion selective electrode with a Clark-type oxygen electrode (Serviddio and Sastre, 2010). However}, however, this method requires millions of cells or large quantities of isolated mitochondria.

$$E = \frac{RT}{zF} \ln \frac{[\text{dye intermembrane space}]}{[\text{dye cytoplasm}]}$$

E = membrane potential in volts

R = ideal gas constant, in joules per kelvin per mole

T = temperature, in kelvins

F = Faraday's constant

z = charge of the dye

$[dye]_{IMS}$ = the intermembrane space dye concentration in moles per m^3

$[dye]_{cyto}$ = the cytoplasmic dye concentration in moles per m^3

Figure 4.1. Nernst equation for distribution of dye and mitochondrial membrane potential ($\Delta\Psi_m$). In thermodynamic equilibrium, where there is no proton flux across the mitochondrial inner membrane, $\Delta\Psi_m$ must be equal to the Nernst potential (E).

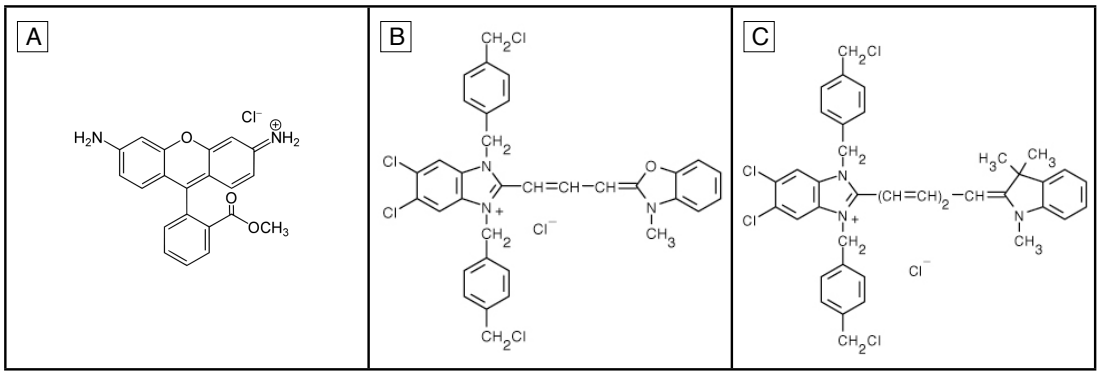

Figure 4.2. Chemical structure of lipophilic fluorescent probes used for visualisation of mitochondria in confocal microscopy. (a) Rhodamine 123, (b) Mitotracker Green® FM, and (c) Mitotracker Red® FM. Rhodamine 123 and Mitotracker Red are known to accumulate in the mitochondria proportionally to their $\Delta\Psi_m$. Interestingly, Mitotracker Green seems to probe mitochondria regardless of their $\Delta\Psi_m$.

Although it has been suggested that developing embryos are always fully dependent on the energy produced by the oocyte mitochondria, mtDNA replication, transcription and biogenesis do not occur until the blastocyst stage (Piko and Taylor, 1987; Taylor and Piko, 1995). Furthermore, for decades now, it has been known that mitochondria from mature oocytes and early cleavage stage embryos are structurally undifferentiated and generate relatively low concentrations of ATP when compared to later stages of development (Plante and King, 1994). Nonetheless, many uncritical studies have persistently associated the quiescent state of oocyte $\Delta\Psi_m$ with infertility and early embryo development failures. Those studies propose that efficiency of mitochondrial respiration is reduced in the oocytes of older women with fertility problems (Wilding, et al., 2001), and that *in vitro* transfer of active mitochondria into oocytes may increase the chances of implantation (Van Blerkom, et al., 1998) and improve embryogenesis (Nagai, et al., 2004). Other studies suggest that developing *Drosophila* oocytes contain specific mechanisms to ensure the supply of highly functional mitochondria (Cox and Spradling, 2003), and that mitochondrial membrane potential and ATP generation is essential to embryo development, mitochondrial integrity, and to sustain fertility in mammalian eggs (Dumollard, 2004; Dumollard, et al., 2006).

Conversely, an opposite view has been hypothesized (Allen, 1996) (reviewed in Section 1.3.5), that the female germ line mitochondria must remain bioenergetically inactive for accurate transmission of maternal mtDNA. In support to this hypothesis, and contrary to what has been said on the subject elsewhere, more recent studies have found that oocyte mitochondrial $\Delta\Psi_m$ tends to elevated in abnormal circumstances, rather than decreased. Domains of "low-polarised" mitochondria have been demonstrated to occur naturally in human and mouse oocytes and early embryos (Van Blerkom, et al., 2002). Another study shows that mouse 2-cell embryos that are arrested in development seem to have a higher mitochondrial $\Delta\Psi_m$ when compared to normally developing embryos, and that increased $\Delta\Psi_m$ may contribute to failure in the early developmental stages (Acton, et al., 2004). In addition, maternal diet-induced obesity in mice was shown to lead to

an increase in oocyte mitochondrial $\Delta\Psi_m$, mtDNA content and biogenesis (Igosheva, et al., 2010). They also showed that ROS was raised while glutathione was depleted and the redox state became more oxidised, suggestive of oxidative stress (Igosheva, et al., 2010). Furthermore, a mouse knockout line for the maternal lethal effect gene *Nlrp5* show premature activation of mitochondrial $\Delta\Psi_m$, which in turn leads to irreversible mitochondrial damage, and the embryos fails to to develop beyond the 2-cell stage (Fernandes, et al., 2012).

In this chapter, I present a comparative analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in the female and male gametes of *Drosophila*, zebrafish and jellyfish.

4.2. Results

4.2.1. Mitochondrial $\Delta\Psi$m is suppressed in jellyfish oocytes

Confocal microscopy was used to visualize the $\Delta\Psi_m$ in gonads of male and female jellyfish. Figure 4.3a shows the bright field image of *Aurelia* ovary, with a scale bar of 25 μm. The bright field also indicates the female gonad diploid tissues and the oocytes. Figure 4.3b shows that mitochondria appear to be green because of their uptake of the fluorescent dye Mitotracker Green FM. Figure 4.3c shows the same view of a sample of *Aurelia* ovarian tissue, this time visualized with Mitotracker Red FM, which reports specifically on the presence of the membrane potential. In the female gonad diploid cells, the pattern of mitochondrial distribution seen with Mitotracker Red (Figure 4.3c) agrees with that seen as the green colour of Mitotracker Green (Figure 4.3b). However, and in contrast, oocyte mitochondria are unstained by Mitotracker Red (Figure 4.3c), suggesting that oocyte mitochondria sustain little or no proton flux driven by membrane potential. Figure 4.3d is an overlay of the images in Figures 4.3b-c, where the red and green colours of Mitotracker combine to show active mitochondria in yellow or orange. Zoomed images of female gonad somatic mitochondria (Figure 4.3e) and oocyte mitocondria (Figure 4.3f) highlight the presence of two distinct populations of mitochondria. Mitochondria with low or absent membrane potential remain green, as Mitotracker Green does not respond to membrane potential. This technique has been previously described in (Pendergrass, et al., 2004).

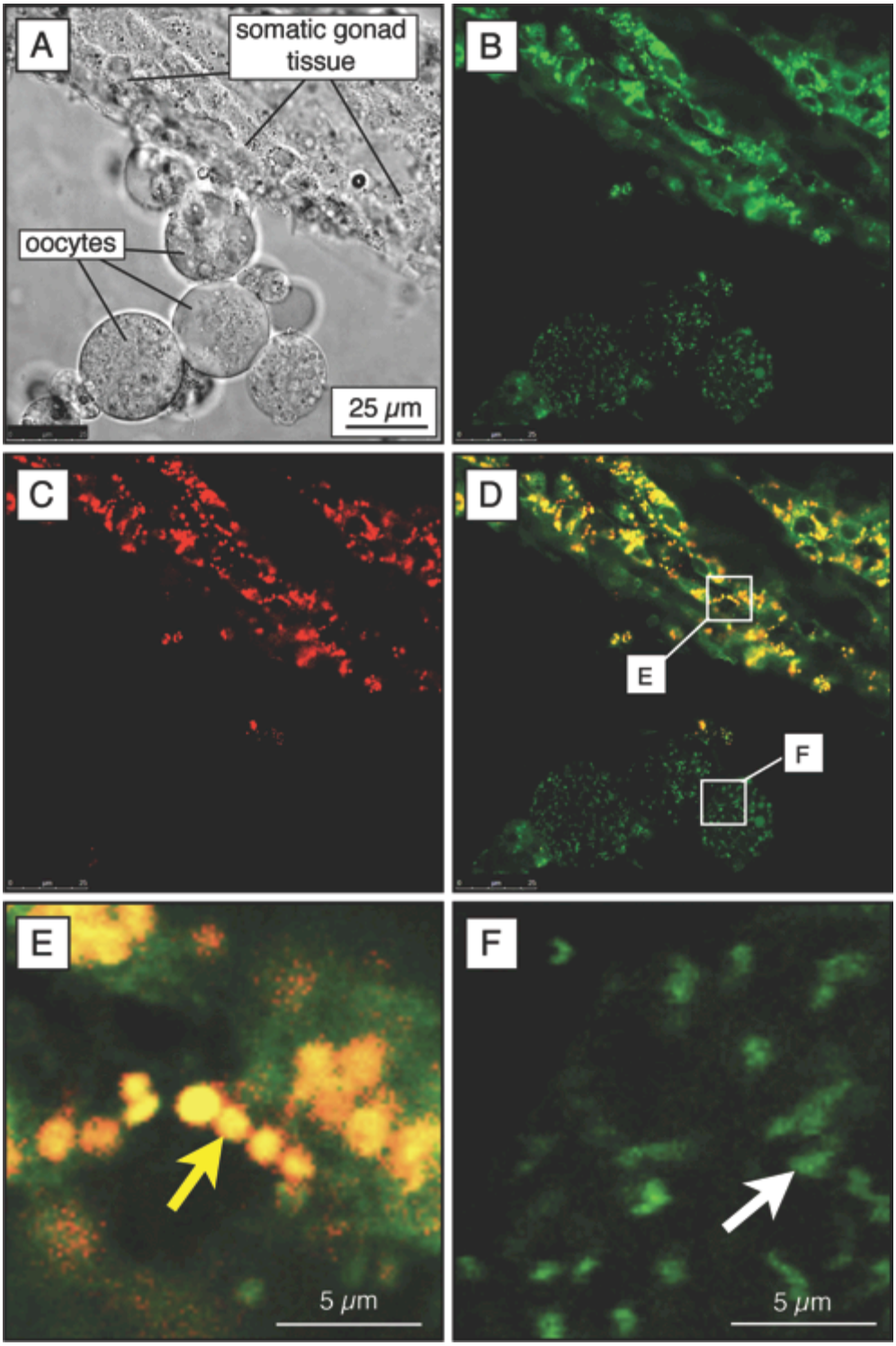

Figure 4.3. Mitochondrial membrane potential in female gonad samples of live *A. aurita*. (a) Confocal light microscopy bright field view, indicating somatic gonad tissue and oocytes, with a scale bar of 25 μm. (b) Mitotracker Green FM (ex/em: 488/520 nm) detects the presence of mitochondria regardless of their $\Delta\Psi_m$. Conversely, Mitotracker Red FM (ex/em: 581/644 nm) is imported into active mitochondria proportionally to their membrane potential (c). Overlay image is shown in (d). Zoomed images from (d) show somatic active mitochondria (e) and quiescent oocyte mitochondria (f), indicated by yellow and white arrows respectively.

Mitochondrial membrane potential was also visualised in *Aurelia* sperm cells as a control. Live *Aurelia* sperm cells, freshly dissected from adult male gonads are shown in the bright field view of confocal light microscopy, with a 25 μm scale bar (Figure 4.4a). Mitotracker Green reveals what seems to be either a large mitochondrion or an agglomerate of a few mitochondria, lying posterior to the nucleus of each cell (Figure 4.4b). A membrane potential-reporting probe, Mitotracker Red, was used to identify which of those mitochondria in figure 4.4b are active and have a proton motive force (Figure 4.4c). Clearly, almost all *Aurelia* sperm mitochondria carry a membrane potential, consistent with their primary role in ATP synthesis, serving sperm motility. An overlay of figures 4.4b and 4.4c shows the most active mitochondria in orange (Figure 4.4d). A zoomed image of figure 4.4a indicates where the sperm nucleus and mitochondria are located (Figure 4.4e). The overlay of Mitotracker Green and Red channels of the same sperm cell highlights an active sperm mitochondria, indicated by a blue arrow (Figure 4.4f).

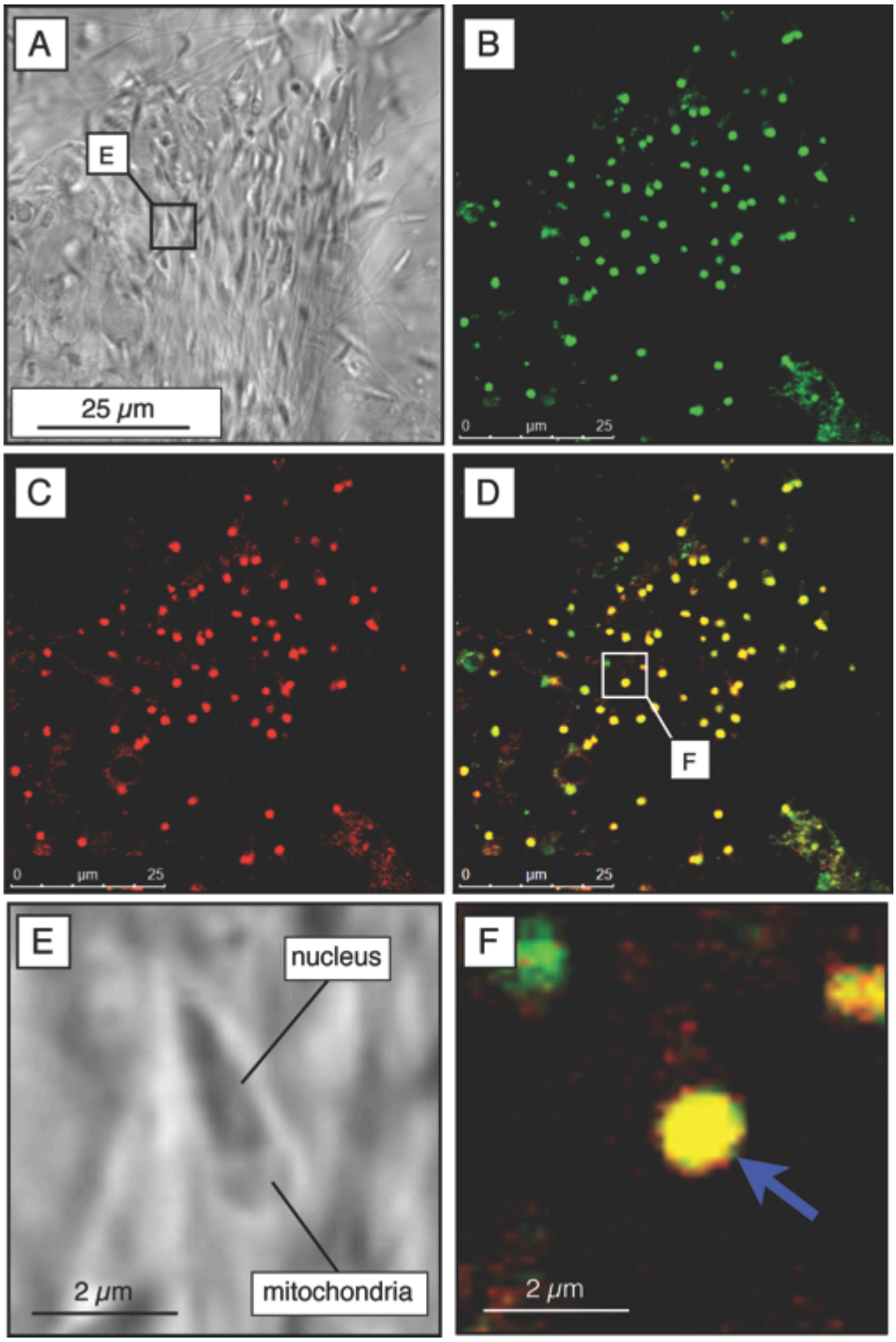

Figure 4.4. Mitochondrial membrane potential of *Aurelia* sperm cells. (a) Bright field view of an *Aurelia* sperm, with a 25 μm scale bar. The same cells were visualized with (b) Mitotracker Green as a mitochondrial marker and (c) Mitotracker Red as mitochondrial membrane potential marker. An overlay of image b,c is shown in (d). Zoomed images of (e) the bright field and (f) both Mitotracker dyes are shown with a scale bar of 2 μm. The blue arrow indicates active sperm mitochondria.

4.2.2. Mitochondrial $\Delta\Psi$m is suppressed in *Drosophila* oocytes

A combination of mitochondrial marker with a proton motive force-reporting dye was also used to investigate the bioenergetic state of *Drosophila melanogaster* female germ line mitochondria (Pendergrass, et al., 2004). Transgenic flies expressing a YFP gene containing a mitochondrial targeting pre-sequence were used in this experiment (LaJeunesse, et al., 2004). Ovaries from live adult female flies were dissected, and different stages of oogenesis (Bastock and St Johnston, 2008) were analysed using confocal light microscopy.

Drosophila germarium and stage 2 eggs are shown in Figure 4.5. In the bright field, a germarium is seen to be giving rise to two Stage 2 eggs (Figure 4.5a). YFP signal co-localises with mitochondria (Figure 4.5b), and Mitotracker Red stains active the ones (Figure 4.5c). The overlay image (Figure 4.5d) shows a group of 4-5 germarium cells (white arrow) that seem to have quiescent mitochondria, while the somatic cells responsible for originating follicle cells in the same germarium (blue arrow) seem to contain only active mitochondria. The large population of quiescent mitochondria seen in the two Stage 2 eggs are likely to have been originated from those germarium cells indicated by the white arrow in Figure 4.5d.

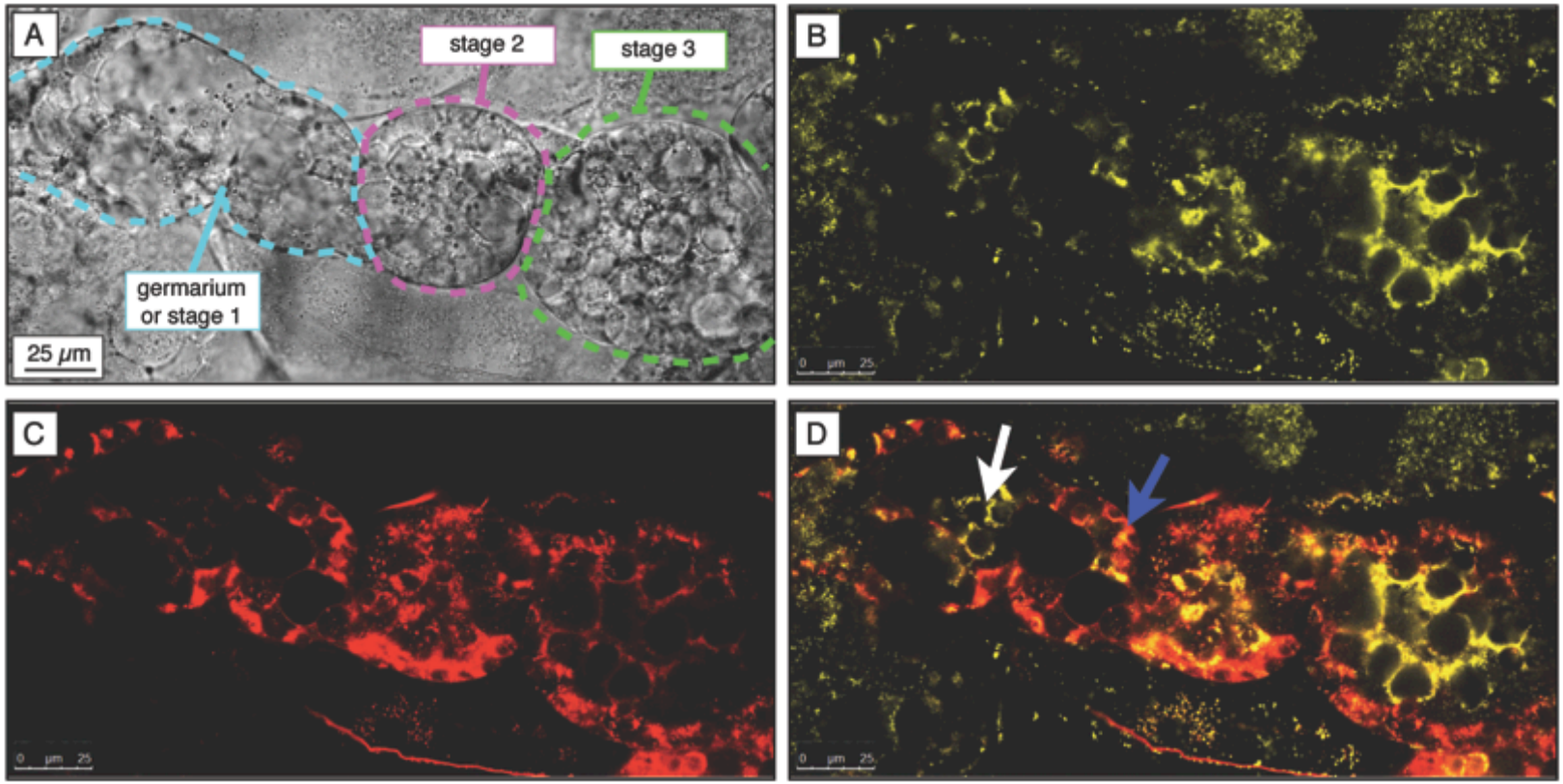

Figure 4.5. Mitochondrial membrane potential in *Drosophila melanogaster* germarium (or stage 1), stage 2, and stage 3 eggs. (a) Bright field shows a germarium (cyan dashed line) giving rise to a stage 2 (magenta dashed line) and a stage 3 egg (green dashed line), with a scale bar of 25 μm. (b) YFP targeted to the mitochondria shows their location in the tissue, regardless of the $\Delta\Psi_m$. (c) Mitotracker red highlights the active mitochondria, and (d) is an overlay of b,c. The white arrow indicates germarium cells containing quiescent mitochondria, which will populate the subsequent stages of oogenesis. The blue arrow indicates the bioenergetically active mitochondria in the follicle-precursor cells.

The ensuing stages of oogenesis in *Drosophila* seem to have the quiescent mitochondria organised in a peri-nuclear clouds, also known as Balbiani bodies. This mitochondrial organisation has been extensively described in the literature, and seems to be present not only in insects (Cox and Spradling, 2003), but also in some vertebrates (Mignotte, et al., 1987). The Balbiani bodies seen in Stage 5 *Drosophila* egg (Figure 4.6) seem to contain clearly two populations of mitochondria: nurse cells and oocytes contain bioenergetically quiescent mitochondria (Figure 4.6f), but not completely depleted of proton motive force (Figure 4.6c), while follicle cell mitochondria (Figure 4.6e) seem to incorporate much more Mitotracker Red into their matrix, which can be directly correlated to their elevated $\Delta\Psi_m$.

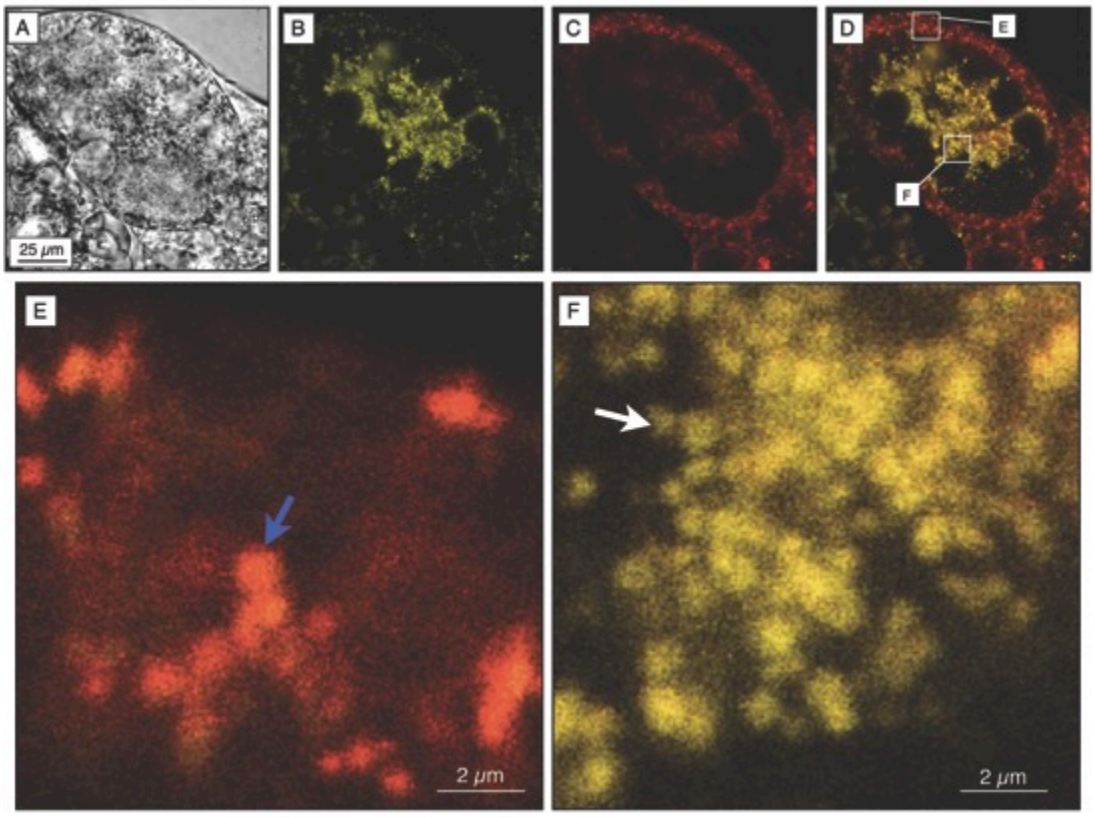

Figure 4.6. Mitochondrial membrane potential in *Drosophila melanogaster* stage 5 egg. (a) bright field a stage 5 (scale bar of 25 μm), (b) mitochondrial marker YFP, (c) Mitotracker red reporting active mitochondria, and (d) is an overlay of b,c. Zoomed images show (e) a highly active individual mitochondrion in follicle cells indicated by a blue arrow, and (f) a quiescent female germ line mitochondria indicated by a white arrow.

More advanced stages of *Drosophila* oogenesis also seem to retain germ line mitochondria in a energetically quiescent state. Stages 9 and 10 (Figure 4.7) are characterised by the degradation of nurse cells, which in turn donate all their mitochondria to a single oocyte (white arrow) via ring canals. Follicle cells also undergo programmed cell death to give rise to the mature egg shell (blue arrows). *Drosophila* female germ line mitochondria seem to consistently retain their ability to suppress their $\Delta\Psi_m$ through oogenesis.

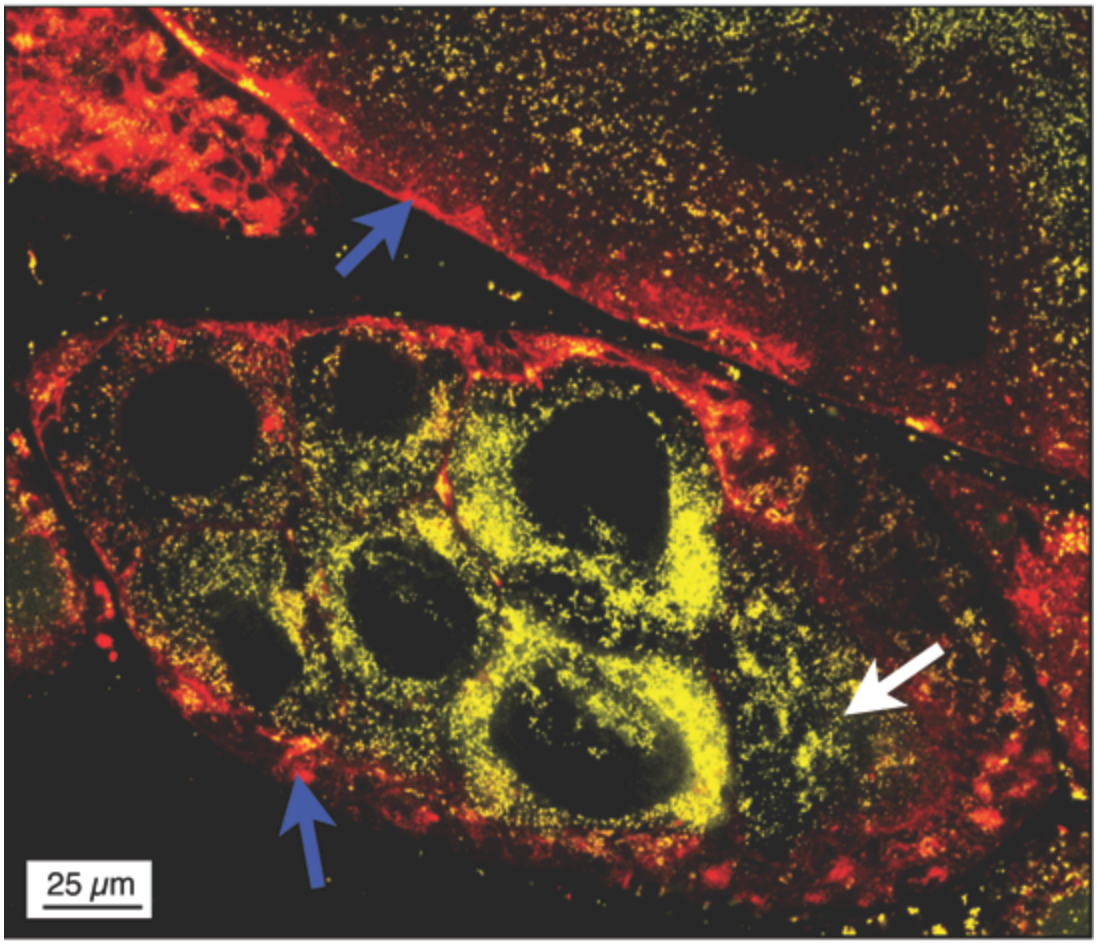

Figure 4.7. Mitochondrial membrane potential in *Drosophila melanogaster* stage 9 (lower) and stage 10 (upper) eggs. The YFP/Mitotracker Red merged image highlights bioenergetically quiescent mitochondria (yellow) moving from surrounding nurse cells undergoing programmed cell death (cells with irregular nucleus shape) toward a single oocyte cell (white arrow). Somatic follicle cells are also undergoing cell death to originate the egg shell (blue arrows), while some residually active mitochondria can still be visualised in the red channel.

To quantify the mitochondrial $\Delta\Psi$ in *Drosophila* egg, the pixel intensity of Mitotracker Red/YFP signal ratio of six independent Stages 5-7 samples was measured. Images were converted to 8-bit, and shades of grey ranging from 0-255 were estimated for per area. Results show that follicle cell mitochondria are likely to have about 50-fold more $\Delta\Psi_m$ than nurse cells and oocytes. Nonetheless, nurse cells and oocyte mitochondria are not completely depolarised, as a basal level of proton motive force was still detected (Figure 4.8).

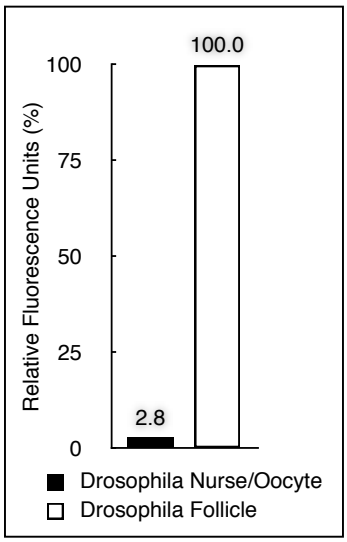

Figure 4.8. Mitotracker Red/YFP ratio, expressed in percentage of relative fluorescence units (RFU), of nurse cells and oocytes in comparison to follicle cells.

A control experiment was done to count the number of mitochondria per cell and per area in germ line cells compared to somatic follicle cells (Figure 4.9). This was done to correct for the possibility that the higher Mitotracker Red fluorescence was detected in follicle cells resulted from a denser mitochondrial population present in those cells, rather than from their elevated $\Delta\Psi_m$. These results show that female germ line cells have more mitochondria per cell compared to follicle cells (Figure 4.9e). Furthermore, when the same comparison is done using number of mitochondria per 625 μm^2, the number of mitochondria in germ cells appears to be almost the same as in the somatic follicle cells (Figure 4.9f). Therefore, it is possible to conclude that the stronger Mitotracker Red fluorescence emitted by follicle cells results from higher $\Delta\Psi_m$, as opposed to a denser population of mitochondria.

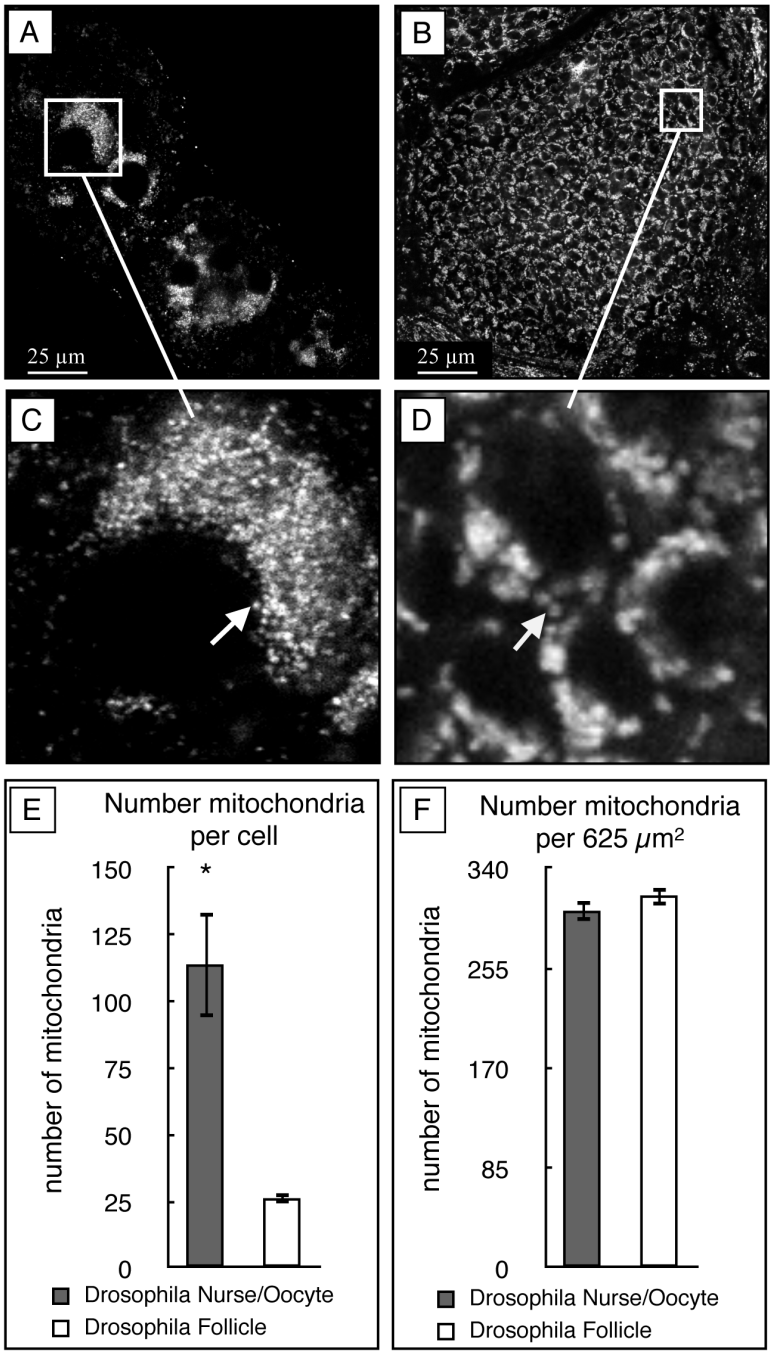

Figure 4.9. Confocal light microscopy of mitochondria in follicle and germ cells of *Drosophila*, using YFP as a mitochondrial marker. (a) *Drosophila* egg, internal view of nurse cells and oocytes and (b) external view of follicle cells. Magnified views of (c) a *Drosophila* nurse cell mitochondrial cloud and (d) a *Drosophila* follicle cell mitochondria. White arrows point to individual mitochondrion. Number of mitochondria per cell and per 625 μm^2 area is shown in (e) and (f) respectively. Error bars represent SEM, generated by three independent biological samples. (*) indicate $P < 0.05$ calculated by paired *t*-test in comparison to follicle cell mitochondrial number.

Drosophila sperm cells were used as a control to show that the quiescence of germ line mitochondria is sex-specific, and applies only to females – which pass on their mitochondria. Testes from adult male flies, from the same transgenic line used in the egg mitochondrial analysis, were dissected to expose the sperm sacs (Figure 4.10a). Sperm sacs were mechanically opened to remove the fly sperm cells (Figure 4.10b) and stain with Mitotracker Red for the mitochondrial membrane potential analysis. Mitochondria are round structures seen along to the length of the sperm tail, as seen in the YFP channel (Figure 4.10c), and those mitochondria appear to be highly active as highlighted in Figures 4.10d and 4.10e.

Drosophila sperm cells are characteristic for their unusual tail length, which can reach sometimes twice the length of an adult fly itself. Another unusual characteristic of sperm cells is their mitochondria, which are highly modified into internal crystalline structures.

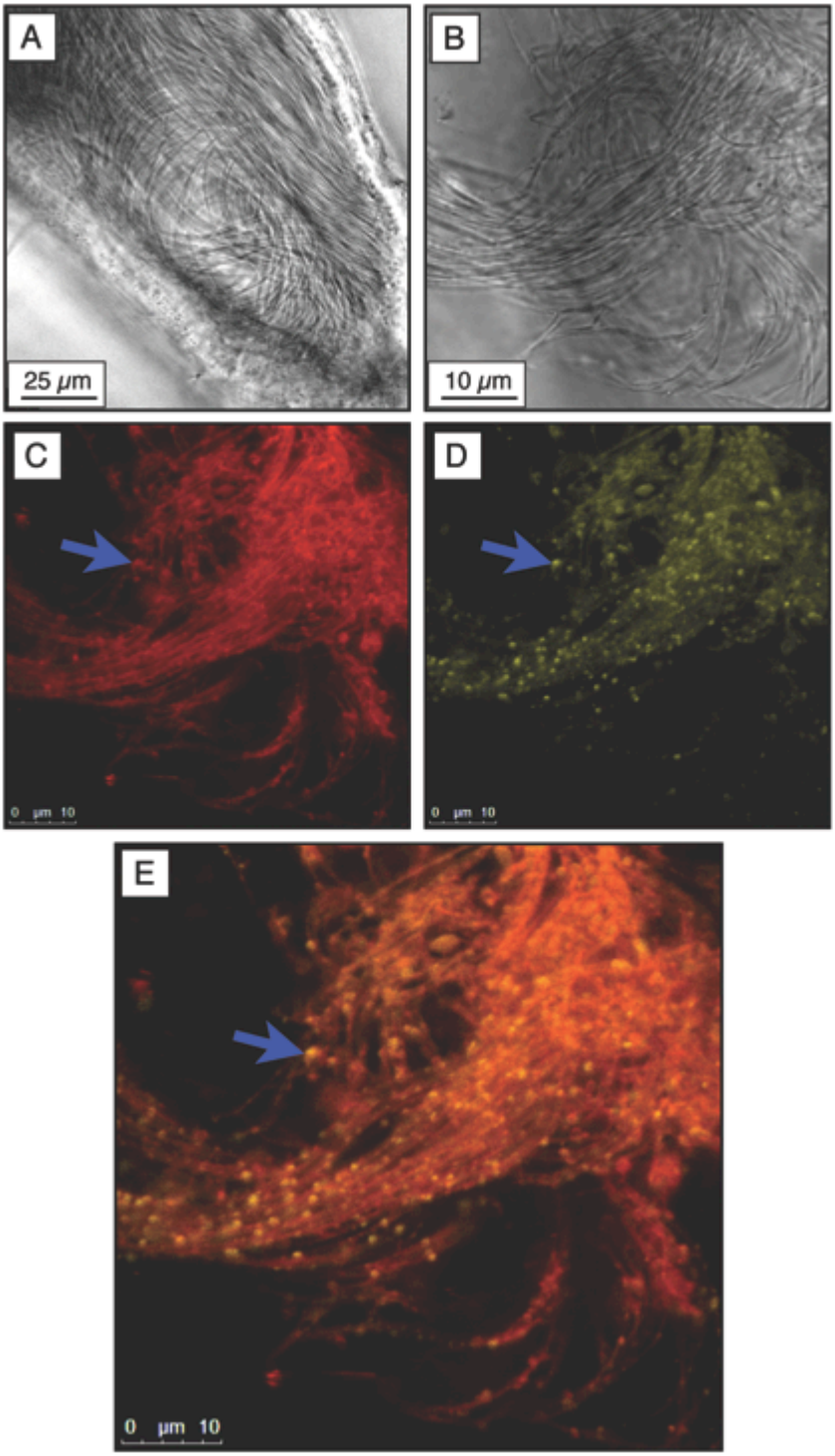

Figure 4.10. Mitochondrial membrane potential in *Drosophila melanogaster* sperm cells. (a) Sperm sacs were dissected from adult male flies and mechanically opened to release (b) individual sperm cells, seen as "hairy" structures in the bright field. (c) YFP co-localised with mitochondria serves as a marker to detect the presence of individual mitochondria, while (d) Mitotracker Red stains those same mitochondria directly proportional to their proton motive force, as pointed by blue arrows. (e) Merged image of (c) and (d) reporting most sperm mitochondria have high $\Delta\Psi_m$.

4.2.3. Mitochondrial $\Delta\Psi$m is suppressed in zebrafish oocytes

An analysis similar to the one described in the previous subsections for jellyfish and *Drosophila* mitochondria was also carried out in somatic, male and female gametic tissues of the zebrafish *Danio rerio*.

Zebrafish ovaries were dissected from live adult wild-type females and subsequently stained with Mitotracker Green and Mitotracker Red, in order to identify distinct populations of high and low-polarised mitochondria (Figure 4.11). Primordial oocytes ranged from 25-50 μm of diameter and are surrounded by a thin layer of small follicle cells (Figure 4.11a). Mitotracker Green reports the location of all mitochondria in those two types of cells, regardless of their $\Delta\Psi_m$ (Figure 4.11b), while Mitotracker Red reports those which are bioenergetically active (Figure 4.11c). If both channels are merged (Figure 4.11d), it is possible to visualise a homogenous population of low-polarised mitochondria, shown in green colour, distributed along the oocyte area. In the other hand, follicle cells contain smaller size mitochondria, nevertheless highly active as seen by strong red coloured speckles distributed around the small follicle cell nucleus.

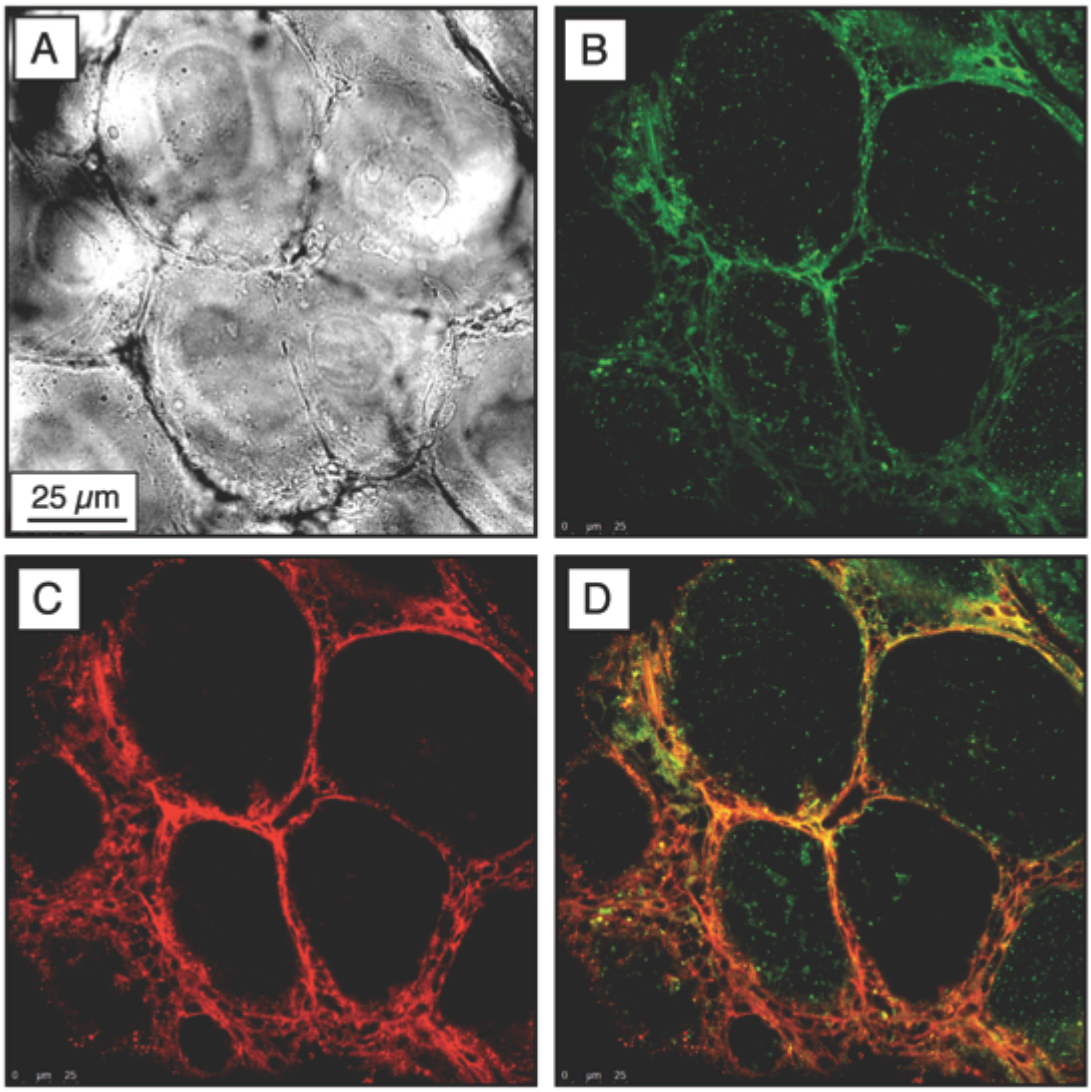

Figure 4.11. Mitochondrial membrane potential of *Danio rerio* primodial oocyte mitochondria. (a) Bright field of an overview of 6-7 primordial oocytes, with a scale bar of 25 μm. (b) Mitotracker Green channel as a mitochondrial marker, and (c) Mitotracker Red reporting the bioenergetically active ones. (d) A merged image of Mitotracker Green and Red highlighting two different populations of mitochondria; the low polarised oocyte mitochondria, and highly active mitochondria in the surrounding follicle cells.

Similarly to Figure 4.8 and 4.9 for *Drosophila*, a control experiment for quantification of Mitotracker Red intensity and number of mitochondria in oocytes compared to follicle cells was performed. Figure 4.12 shows the mean pixel intensity ratio of Mitotracker Red/Mitotracker Green in six independent primordial oocyte samples of zebrafish ovary. The ratio was found to be even lower than the ratio found in *Drosophila* samples, but once again, not completely absent, as a basal proton motive force was still detected in zebrafish oocytes (Figure 4.12).

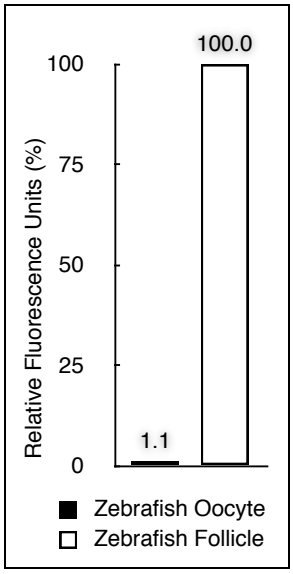

Figure 4.12. Mitotracker Red/Mitotracker Green ratio, expressed in percentage of relative fluorescence units (RFU), of zebrafish oocytes in comparison to their surrounding follicle cells. Follicle cells were set as 100% for comparison.

Even though it is difficult to calculate the exact number of mitochondria in a given oocyte or follicle cell, mainly due to the limitations of the bi-dimensional plane of focus obtained with this microscopic technique, estimates of the number of mitochondria per cell and per 625 μm^2 area of zebrafish primordial oocyte and follicle cells are presented in Figure 4.13. Similarly to *Drosophila*, the number of mitochondria per oocyte is much larger than in follicle cells (Figure 4.13d), probably because oocytes are also much larger in area. However, when the number of mitochondria was estimated per units of area, follicle cells appear to have three times more mitochondria than oocytes (Figure 4.13e). This may still not account for the 100-fold higher intensity of Mitotracker Red fluorescence in those cells compared to oocytes, suggesting higher $\Delta\Psi_m$, rather than a larger number of mitochondria per units of area.

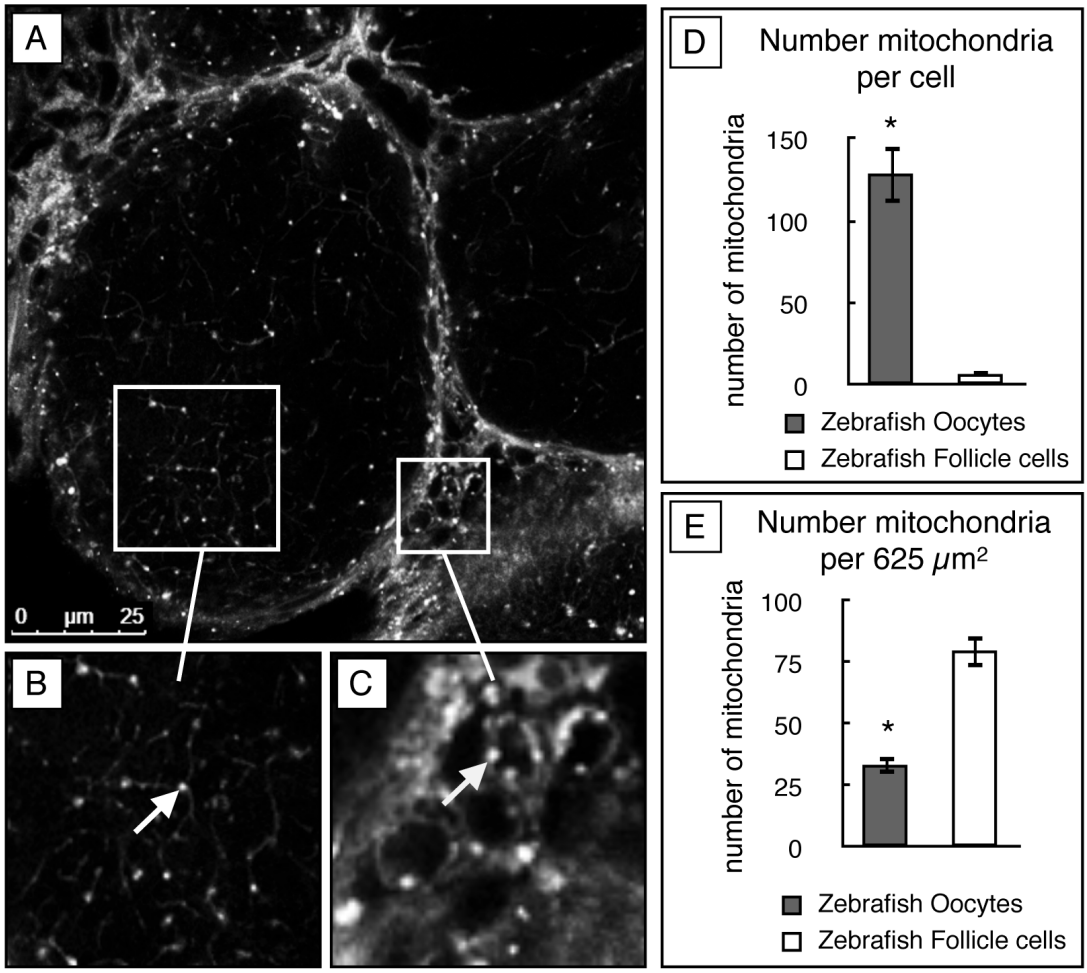

Figure 4.13. Confocal light microscopy of mitochondria in follicle and germ cells of zebrafish, using Mitotracker Green as a mitochondrial marker. (a) Primordial oocytes and their surrounding follicle cells. Magnified views of (b) a population of primordial oocyte mitochondria and (c) follicle cell mitochondria. White arrows point to individual mitochondrion. Number of mitochondria per cell and per 625 μm^2 area is shown in (d) and (e) respectively. Error bars represent SEM, generated by three independent biological samples. (*) indicate $P < 0.05$ calculated by paired t-test in comparison to follicle cell mitochondrial number.

To find out whether mitochondria are quiescent in both male and female gametes, or only in the female germ line, adult male zebrafish at their reproductive stages were dissected and their testes removed for mitochondrial $\Delta\Psi$ imaging of sperm cells (Figure 4.14). Zebrafish sperm mitochondria are seen as triangular structures in the posterior part of a round nucleus. Not surprisingly, highly motile zebrafish sperm exhibited a strong Mitotracker Red fluorescence signal, clearly seen in (Figure 4.14c,g).

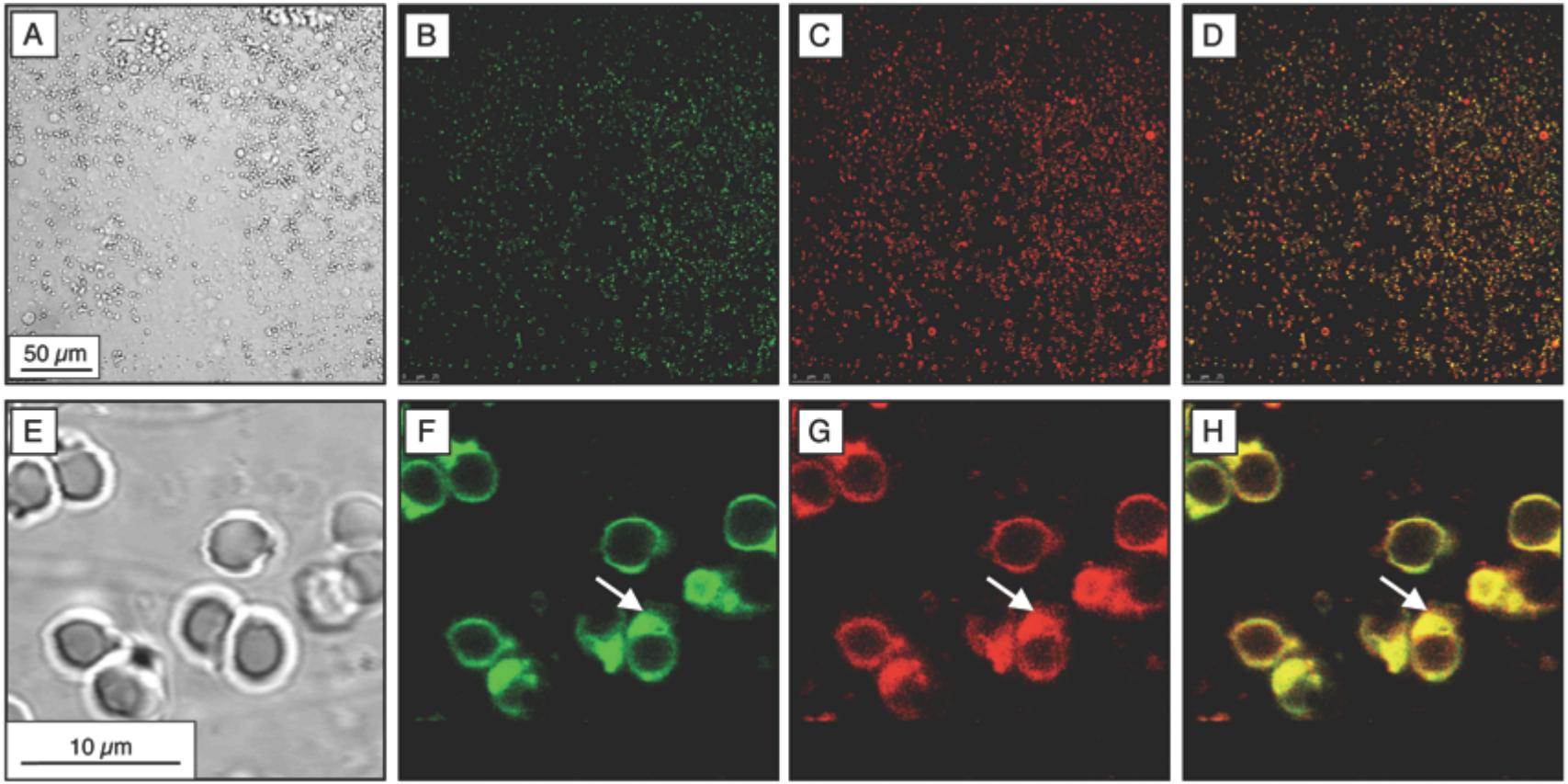

Figure 4.14. Mitochondrial $\Delta\Psi$ of zebrafish sperm cells. (a) Bright field showing tens of thousands live sperm cells released from freshly dissected zebrafish testis, seen also under (b) Mitotracker Green and (c) Mitotracker Red and (d) an overlay of both channels. (e) Bright field of a closer view of three sperm cells, with a scale bar of 10 μm. (f) Mitotracker Green reveals their mitochondria, and (g) Mitotracker Red reports their $\Delta\Psi_m$, further highlighted in the overlay of both channels in (h). White arrows point to sperm mitochondria.

4.3. Discussion

To interpret these results correctly, it is important to note that, because of the hyperbolic nature of the $\Delta\Psi_m$ /respiration rate relationship, $\Delta\Psi_m$ does not necessarily reflect the rate of oxygen consumption in mitochondria (Dobbs and Moore, 1987). However, although mitochondria are able to sustain a $\Delta\Psi$ without any electron transport, the ionic conductance of the inner membrane is directly dependent on the $\Delta\Psi_m$ (Dobbs and Moore, 1987). Therefore, it is plausible to assume that if $\Delta\Psi_m$ is suppressed or uncoupled by any given mechanism, the inner membrane conductance would be consequently restricted, thus affecting the rate of electron transport and oxygen consumption.

Taken together, the results presented in this chapter leave little doubt that oocyte mitochondria have a much lower membrane potential when compared to other somatic tissues and sperm cells in the case of jellyfish, *Drosophila* and zebrafish. Suppression of mtDNA transcription (Chapter 3) may be one of the reasons why oocytes maintain such low $\Delta\Psi_m$. This phenomenon seems to be a natural mechanism that occurs consistently in healthy individuals of these three species, which diverged over 500 million years ago, rather than as a result of oocyte developmental incompetence or pathological fertility issues as previously proposed for mammals (Chappel, 2013; Dumollard, 2004; Hsieh, et al., 2004). But if that is the case, how can oocytes survive without a source of ATP? Furthermore, depolarised mitochondria are targeted to mitophagy via Parkin recruitment (Sarraf, et al., 2013), and are also incapable of sustaining membrane-potential driven protein import (Geissler, et al., 2000). How do oocytes circumvent these problems?

There is clear evidence that ATP is required for oocyte maturation, especially during meiosis resumption where energy is a critical factor for efficient spindle formation and chromosome segregation (Stojkovic, et al., 2001; Van Blerkom, et al., 1995). If the oocyte does not synthesise its own ATP via oxidative phosphorylation, then it has to depend largely either on glucose metabolism, or in an exogenous supply of ATP. Interestingly, both seem to be case.

In amphibians, oocyte glycolysis has been shown to be active, and significant ATP production was detected in the presence of 0.1 mM cyanide (Guixe, et al., 1994). Other studies have demonstrated that oocytes from a pyruvate dehydrogenase (PDH) mouse-knockout line can successfully grow to normal size, be ovulated, and fertilized, which strongly suggests that oocytes do not rely on oxidative phosphorylation for those functions (Johnson, et al., 2007). However, the embryo could not develop beyond single-cell stage in those mutants, mainly due to severe meiotic dysfunction, suggesting the pyruvate metabolism might still be associated with embryonic developmental competence (Johnson, et al., 2007). Other work suggests that not only glycolysis is active, but also the rate-limiting enzyme of the oxidative pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase, is abundantly active in oocytes, while it decreases significantly with development (Krisher and Bavister, 1999). In addition, resumption of meiosis has been shown to be associated with elevated activity of glycolysis and enzymes (Krisher and Herrick, 2013).

Besides relying on glucose metabolism for ATP production, female germ cells also seem to receive high concentrations of ATP from their surrounding follicle cells. As seen in the results presented here for *Drosophila* and zebrafish, somatic follicle cells possess highly active mitochondria, with exception of the jellyfish, which does not have this special layer of cells, apparently specialised in oxidative phosphorylation. The mechanism by which oocytes obtain high amounts of ATP concentrations from their surrounding somatic cells seems to be achieved by gap-junctions, which are cellular connections projected from the base of follicle cells into the oocyte cytoplasm (Albertini and Anderson, 1974; Anderson and Albertini, 1976). Interestingly, experimental work has shown not only that ATP is able to traverse follicle cells into the oocyte via these junctions, but also that hormones and other signaling molecules, which are key to oocyte maturation (Webb, et al., 2002). Additionally, mouse oocytes enclosed by follicle cells were shown to have much higher concentrations of ATP, when compared to naked oocytes (Dalton, 2011), as previously suggested in the work reviewed by (Van Blerkom, 2011). In summary, it is

reasonable to conclude that oocytes rely largely on glucose metabolism and an exogenous source of ATP to maintain its ATP homeostasis, while suppressing the respiratory electron transport chain to maintain the integrity of its mitochondrial DNA.

Another question is: How do oocyte mitochondria escape mitophagy, which is normally triggered by membrane depolarisation? Another important and subtle piece of evidence presented here is that the oocyte mitochondria are not completely depolarised. Somatic cells show 50-100-fold more Mitotracker Red fluorescence compared to oocytes, nevertheless a basal proton motive force was still detected. This may explain how oocyte mitochondria are not targeted for degradation, and furthermore, this may also explain how the oocyte mitochondria can still import most of their protein precursors, which are synthesised by cytosolic ribosomes. But then, another question arises: how can oocytes maintain a basal membrane potential without oxidative phosphorylation? One explanation might lie on the versatile properties of the mitochondrial F_1F_0-ATP synthase. This protein complex can not only synthesise ATP, but also hydrolyse it to generate a proton gradient across the membrane (Fillingame, 1990; Stock, et al., 1999), reviewed by (Weber and Senior, 2000). Therefore, a possibility would be if the oocyte mitochondrial F_1F_0-ATP synthase is regulated in a way that it hydrolyses some of the ATP supplied by surrounding follicle cells and anaerobic metabolism, in order to maintain a basal proton gradient across their membrane. To date there is no experimental evidence for this possibility, but will definitely be an exciting future direction for the work presented in this chapter.

5

Mitochondrial reactive oxygen species in the female germ line

In 2003, the American gerontologist Denham Harman said during an interview (Harman and Harman, 2003):

"*I think the future is great. But I hope that scientists will start looking at the real problem, finding practical ways to slow the innate aging process. People have been measuring this and that as a function of age, or they have tried to minimize disorders, etc, that shorten life. But that doesn't get down to the innate aging problem. The importance of slowing the innate aging process cannot be overstated! We need people who can do more than just turn the crank.*"

5.1. Introduction

Energy-conserving aerobic respiratory electron transport terminates with concerted four-electron reduction of molecular oxygen to give two molecules of water, a reaction usually coupled to proton translocation across the inner membrane and catalysed by the enzyme cytochrome c oxidase. However, molecular oxygen is also readily reduced, at a number of different points in the respiratory chain, by transfer of a single electron, in which case the product is the superoxide anion radical, $O_2^{\bullet -}$ (Allen and Raven, 1996; Chance, et al., 1979; Chen, et al., 2003; Kussmaul and Hirst, 2006; Ohnishi, et al., 2010). Superoxide is a short-lived free radical that reacts rapidly with any of a wide range of chemical substrates, including itself. Superoxide engages spontaneously in disproportionation, or dismutation, a reaction that is also catalysed by the enzyme superoxide dismutase (McCord and Fridovich, 1969). Superoxide dismutase is ubiquitous in aerobic organisms and also present in many anaerobes; in all cases, it is thought to provide a degree of protection from oxygen toxicity (Li, et al., 1995; Melov, et al., 1998; Taufer, et al., 2005; Treiber, et al., 2011). The products of the reaction are oxygen and peroxide, O_2^{2-}, which becomes protonated to give hydrogen peroxide, H_2O_2, at neutral pH.

These and other ROS chemically modify cellular constituents, including lipids (Pessayre, et al., 2001), proteins (Berlett and Stadtman, 1997) and nucleic acids, the latter including production of thymidine dimers (Cooke, et al., 2003). Because of those reactions, the univalent reduction of oxygen is both cytotoxic and mutagenic. Where ROS react with mtDNA, the result may be a modified respiratory chain protein that becomes more prone to univalent reduction of oxygen, thus increasing the frequency of mutation. In this way, it is proposed that a vicious circle of self-augmenting mutational load in mitochondrial DNA may be a primary cause of ageing and of many of its associated degenerative diseases (Ames, et al., 1993; Ames, et al., 1995; Cooke, et al., 2003; Harman, 1972; Harman, 1992; Shigenaga, et al., 1994), including cancer (Petros, et al., 2005).

It is, however, still debated whether the mitochondrial theory of ageing, as previously proposed, is central to the ageing process, part of a multi-factorial mechanism, or perhaps not related to ageing at all (Afanas'ev, 2010; Jacobs, 2003). Some studies suggest that ageing-related mtDNA mutations may arise from DNA replication errors rather than ROS-induced damage (Park and Larsson, 2011), which may subsequently trigger downstream apoptotic markers rather than ROS production (Kujoth, et al., 2005). Other work proposes that ageing processes induced by mtDNA mutations do not involve ROS production (Trifunovic, et al., 2005; Trifunovic, et al., 2004). Conversely, recent studies support the mitochondrial theory of ageing, as reviewed in more detail in section 1.2.2.

As a solution to this problem, it has been proposed that oocytes contain genetically suppressed, template mitochondria that are retained within the female germ line to be transmitted, in the oocyte cytoplasm, between generations (Allen, 1996). According to this hypothesis, sperm mitochondria are subject to ROS-induced mutagenesis since they are required to produce ATP for motility. If sperm mitochondrial DNA were retained, after fertilization, by the zygote, then males would transmit damaged mitochondrial DNA to their offspring. Male gametes and somatic cells of both sexes are predicted to contain energetically functional mitochondria, active in transcription of their DNA. In contrast, oocytes are predicted to obtain ATP by fermentation, by anaerobic respiration, or by import from neighbouring somatic cells, as previously discussed in section 4.3.

To tackle this question, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used as a ROS marker in live specimens. It is thought that, after passing through the plasma membrane, this lipophilic and non-fluorescent compound is de-esterified onto a hydrophilic alcohol (H_2DCF), which is then oxidized by cellular ROS giving rise to the green fluorescent DCF (Karlsson, et al., 2010).

5.2. Results

5.2.1. ROS production is low in jellyfish oocytes

To estimate how much ROS is produced in jellyfish oocytes, female gonads were dissected from live adults female jellyfish medusa, and subsequently stained with H_2DCF-DA to reveal sites of ROS production, according to the procedure described by (Owusu-Ansah, et al., 2008). If ROS is present, the oxidised form of H_2DCF-DA will emit a green fluorescence at the centred at 520 nm, whereas, if it remains reduced, no fluorescence will be observed. Figure 5.1a shows jellyfish ovary in bright field confocal light microscopy. Figure 5.1b shows the same tissue where the blue colour arises from 4',6'-diamidino-2'-phenylindole dihydrochloride (DAPI), a specific stain for double-stranded DNA. Figure 5.1c shows green fluorescence from the oxidized form of H_2DCF-DA, reporting on production of ROS. Figure 5.1d is an overlay of figure 5.1b,c, and suggests that the oocyte (within white dashed lines) generate little or no ROS. Conversely, surrounding gonad diploid cells seem to produce enough ROS to oxidize H_2DCF-DA, which emits the observed green fluorescence.

Similarly, another image shows jellyfish female gonads with eggs stained with H_2DCF-DA for ROS detection (Figure 5.2), but at this time an over-exposed image of the green channel highlights the presence of very small quantities of ROS in the oocyte mitochondria (white arrow), suggesting that the oocyte mitochondria is not completely depleted of ROS (Figure 5.2e).

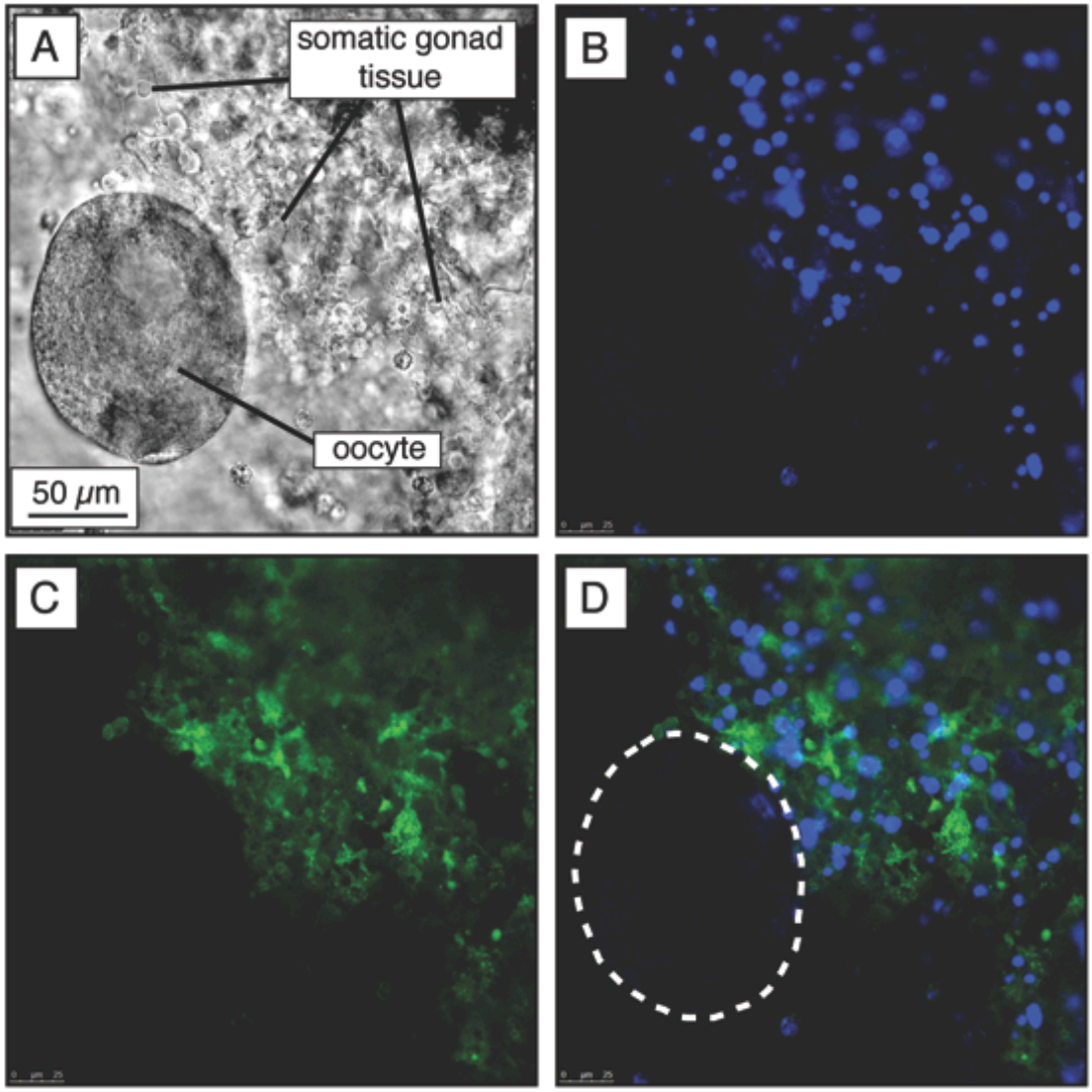

Figure 5.1. ROS production in female gonads of the jellyfish *Aurelia aurita*. (a) Bright field shows a mature jellyfish oocyte of approximately 100 μm of diameter (left), with surrounding somatic gonad tissue (right). (b) DAPI channel detecting the presence of DNA. (c) H_2DCF-DA reporting on the production of ROS in those cells. (d) Overlay image of b, c, highlighting the presence of ROS in the somatic cells, while the oocytes emits undetectable amounts of green fluorescence. White dashed line delimits the egg area.

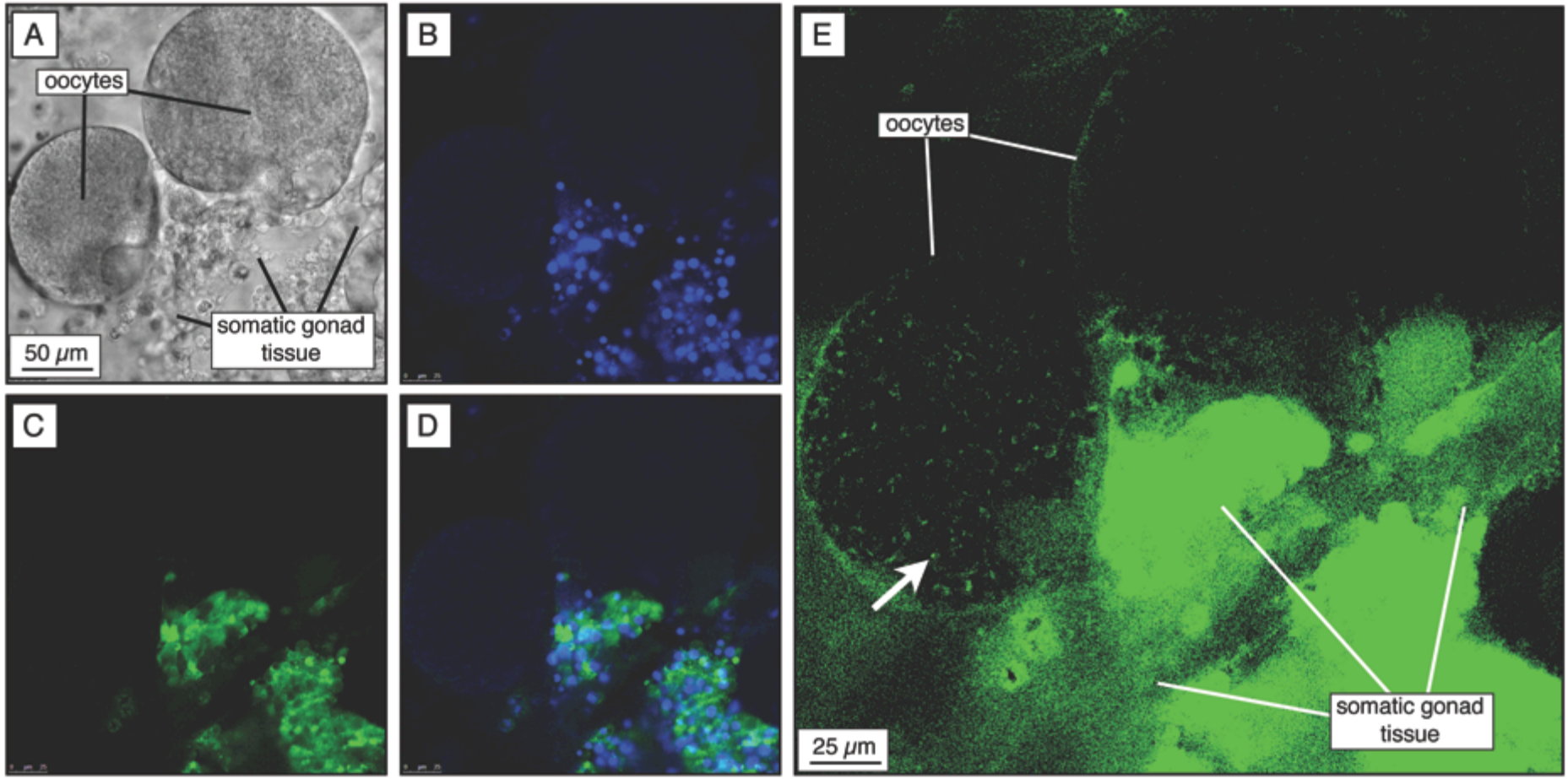

Figure 5.2. ROS production in female gonads of the jellyfish. (a) Bright field shows two oocytes with surrounding somatic gonad tissue. (b) DAPI reveals DNA and (c) H_2DCF-DA reveals ROS in the tissue. (d) Overlay image of b and c. (e) Increased exposure of the image shown in (c), which reveals oocyte mitochondria (white arrow), indicating that oocyte mitochondria may still produce very small quantities of ROS.

A control experiment to assess the ROS production in jellyfish sperm mitochondria was performed, as described for oocytes (Figure 5.3). Jellyfish sperm has a characteristic aggregate of large mitochondria posterior to a triangular-shaped nucleus (Figure 5.3a). Those mitochondria were found to produce large amounts of ROS as seen by the green fluorescence emitted by the oxidised form of H_2DCF-DA (Figure 5.3c).

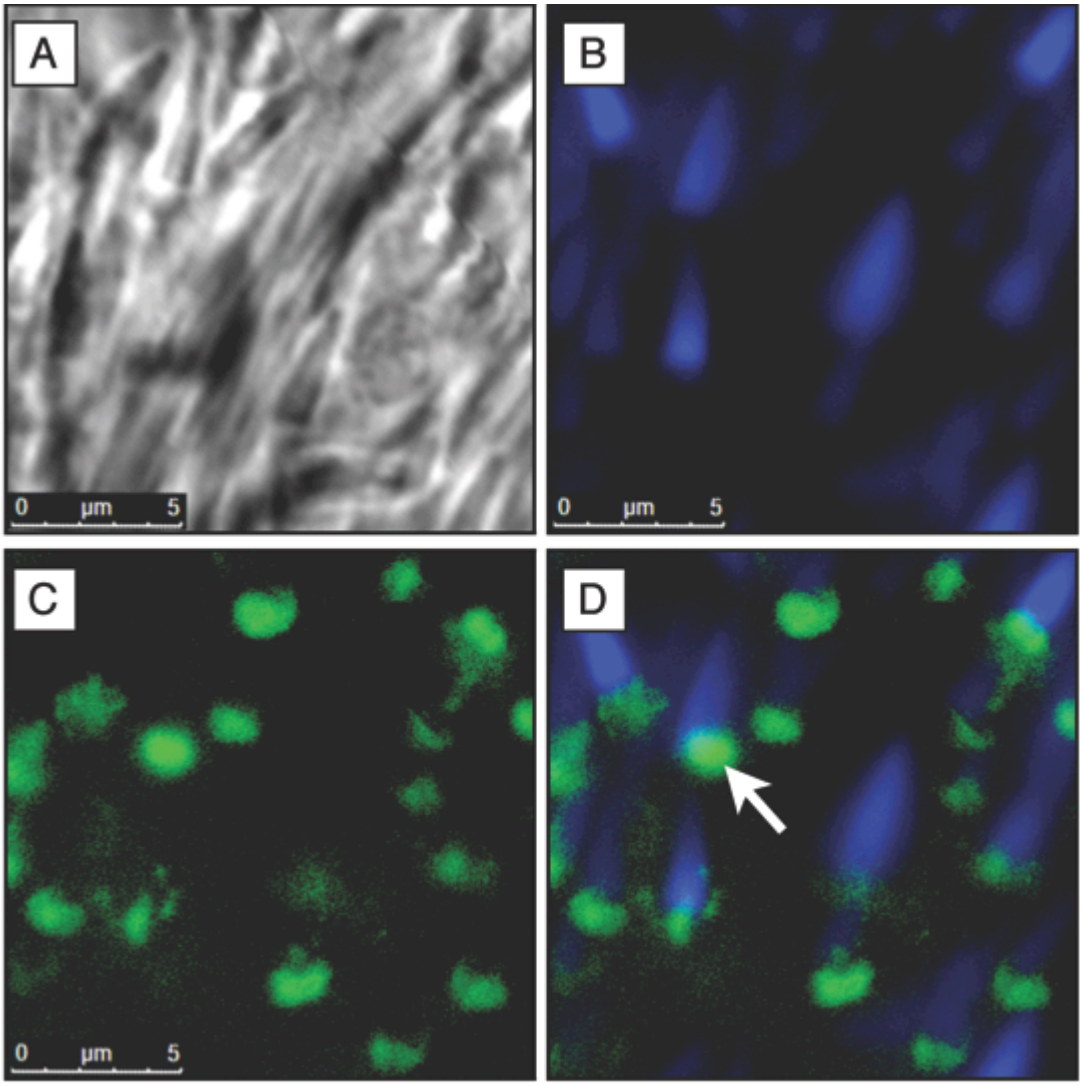

Figure 5.3. ROS production in jellyfish sperm cells. (a) Bright field shows several sperm cells. (b) DAPI reveals the triangular-shaped sperm nuclear DNA and (c) shows the green fluorescence emitted by the oxidised form of H_2DCF-DA indicating the presence of ROS in those cells. (d) Overlay image of b and c, indicating that the ROS produced in likely to come only from mitochondria, which is located in the mid-piece posterior the sperm nucleus. The white arrow points to sperm mitochondria.

5.2.2. ROS production is low in *Drosophila* oocytes and nurse cells

To estimate the production of ROS by female germ cells, *Drosophila* eggs at different stages were stained with DAPI, visualizing DNA as blue, and with the ROS indicator H_2DCF-DA, similarly as described earlier in this chapter for the jellyfish samples. Within ovaries of *Drosophila* (Figure 5.4), somatic follicle cells show higher H_2DCF-DA fluorescence than the female germ cells that they surround.

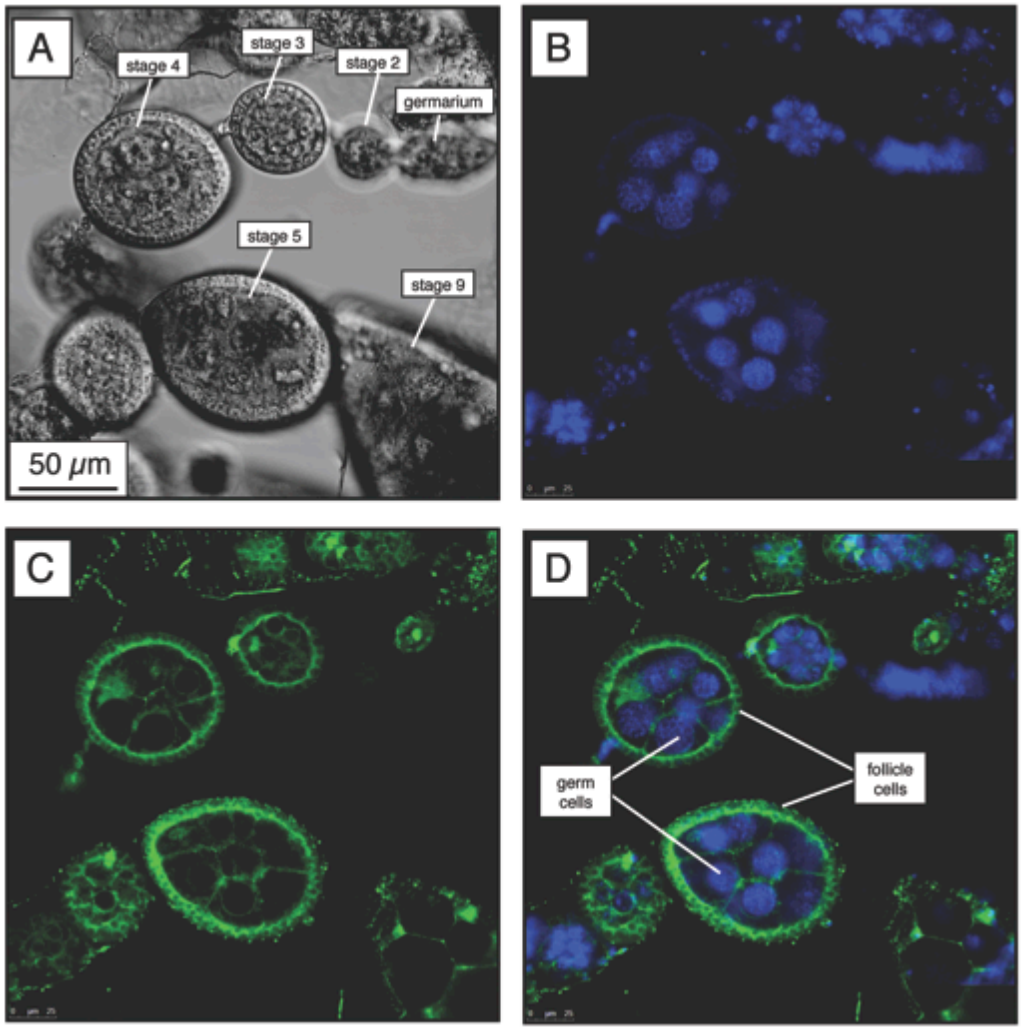

Figure 5.4. ROS accumulation in different *Drosophila* egg stages. (a) The bright field micrograph indicates the stages and corresponding scale bar. (b) DAPI indicates nuclear DNA in the blue channel. (c) Oxidised H_2DCF-DA is seen in the green channel, and reports the relative amount of ROS in different tissues. (d) Merged overlay of both channels highlights the abundance of ROS in somatic follicle cells compared to the female germ line cells, suggesting a reduced rate of electron transfer to oxygen in this cell type.

Another noteworthy characteristic of ROS production in *Drosophila* ovaries is that ROS tend to accumulate more in the basal region of follicle cells (Figure 5.5c; magenta arrows), which is the site where gap-junctions connect them with the oocyte and nurse cells. Nevertheless, a minute quantity of ROS was still detected in the female germ cell mitochondria (Figure 5.5c,d; white arrow). Further analysis of the intensity of which H_2DCF-DA emits from those *Drosophila* ovary samples was measured and quantified in pixel intensity of 8-bit images, expressed in a graph of percentage of relative fluorescence units (Figure 5.5e).

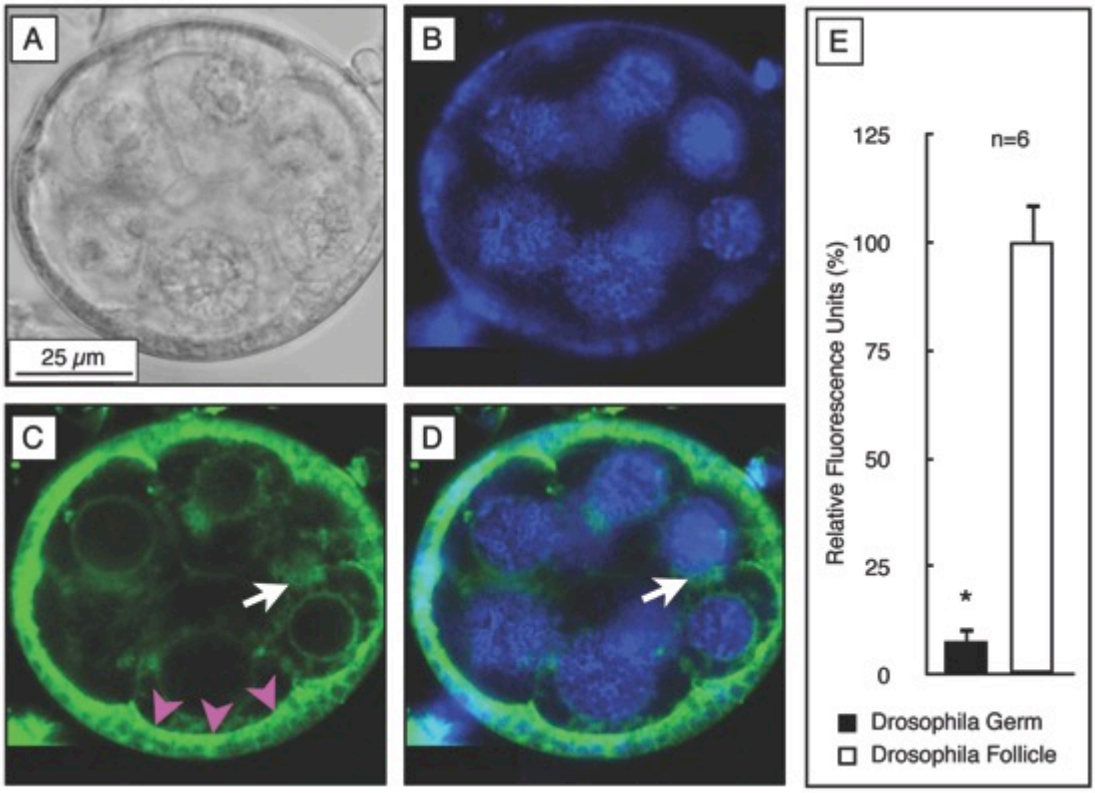

Figure 5.5. Accumulation of ROS in the basal region of follicle cells. (a) Bright field shows a *Drosophila* egg at stage 3. (b) DAPI stained nuclei, followed by (c) the green fluorescence reporting oxidised H_2DCF-DA. Magenta arrows indicate the accumulation of ROS in the basal region of somatic follicle cells. (d) An overlay of (b) and (c). White arrows point to germ cell mitochondria. (e) Graph show the relative mean intensities of H_2DCF-DA fluorescence of oocyte and nurse cells in comparison to follicle cells, set to 100% for normalisation. Six independent biological samples were measured. Error bars were generated by S.E.M. (*) indicates $P<0.05$.

Drosophila sperm cells, dissected from adult male flies, were also analysed with the same method as described for ovaries. Those cells were seen with high concentrations of ROS throughout the length of the tail, mostly concentrated in tiny speckles, which seem to be mitochondrially-derived paracrystalline structures (Figure 5.6d; white arrow). ROS production is a lot more reduced in the female germ cells, when compared to somatic and sperm cells, which correlates results showing suppression in mtDNA transcription (Chapter 3) and mitochondrial membrane potential (Chapter 4).

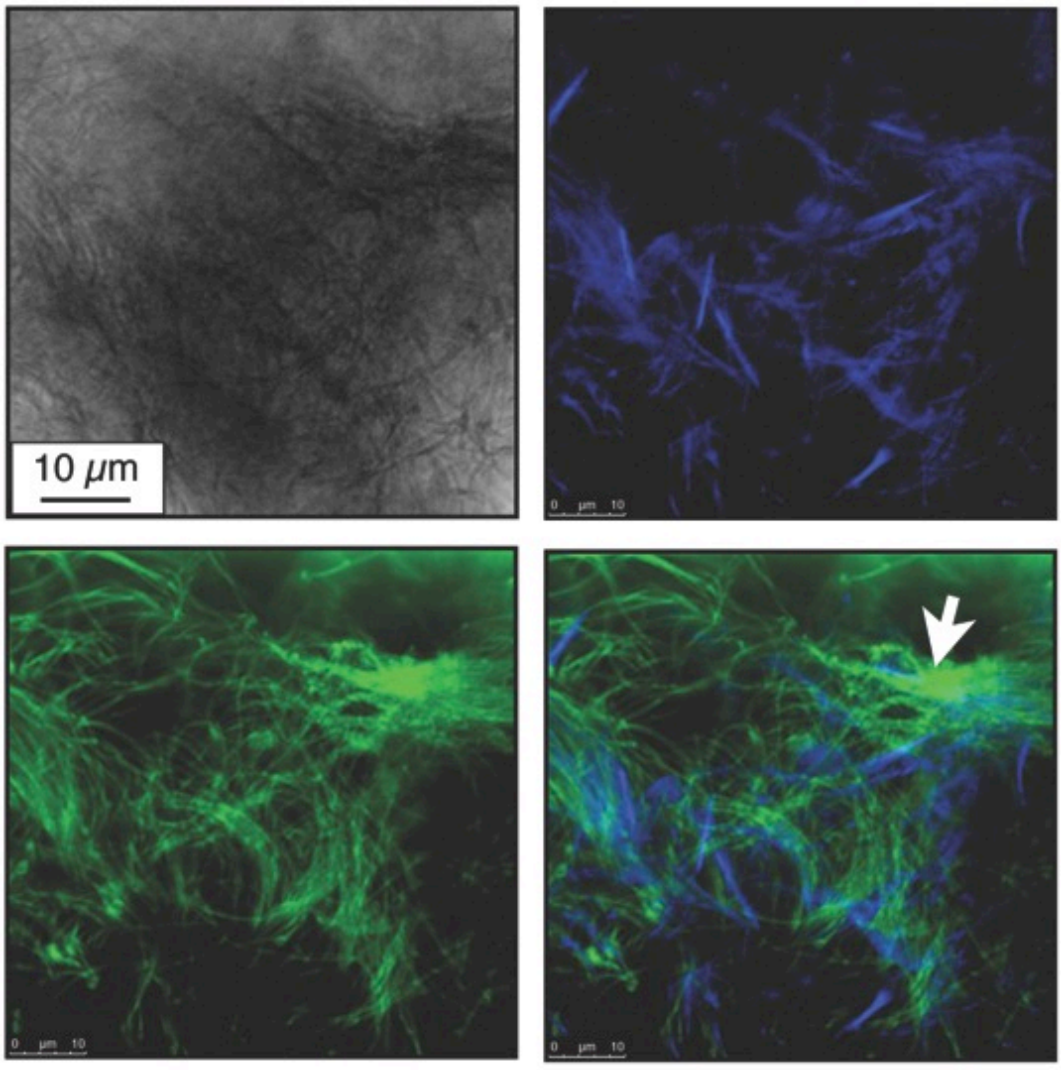

Figure 5.6. ROS accumulation in *Drosophila* sperm cells. (a) The bright field shows sperm cells as hair-like structures. (b) DAPI channels shows the nucleus of each sperm cells in blue, and (c) H_2DCF-DA green fluorescence reports ROS. (d) An overlay image of (b) and (c), with a white arrow pointing to tiny speckles, which seem to be paracrystalline structures derived from mitochondria, and a major source of ROS in those cells.

5.2.3. ROS production is low in zebrafish oocytes

ROS production was also visualised in zebrafish oocytes, using the same technique described earlier for jellyfish and *Drosophila* oocytes (Owusu-Ansah, et al., 2008). Adult wild-type female *Danio rerio* were dissected to remove the ovaries, which were immediately stained with H_2DCF-DA and DAPI (Figure 5.7). Primary oocyte, cortical alveoli and mature oocyte stages are shown in the bright field (Figure 5.7a,e). DAPI is seen to stain most of small nuclei of the follicle cells layer, while oocyte nuclei are more difficult to detected, as DAPI fluorescence signal tends to dissipate in tissue depth higher than ~60 μm. H_2DCF-DA is seen highly abundant in the follicle cells of mature oocytes, while cortical alveoli developing oocytes show a thin layer of follicle cells that already accumulate some ROS (Figure 5.7c,g). In contrast to the results obtained with jellyfish and *Drosophila* samples, oocyte mitochondria could not be detected using H_2DCF-DA. The overlay images show DAPI plus H2DCF-DA, and highlight the abundance of ROS in the somatic follicle cells (Figure 5.7d,h). Further quantification measurements were carried out in six independent zebrafish oocyte samples, to determine the intensity of the green fluorescence emitted by oxidised H_2DCF-DA in oocytes, when compared to the somatic follicle cells (Figure 5.7i). Oocytes show approximately 100-fold less ROS accumulation when compared to the more active somatic follicle cells.

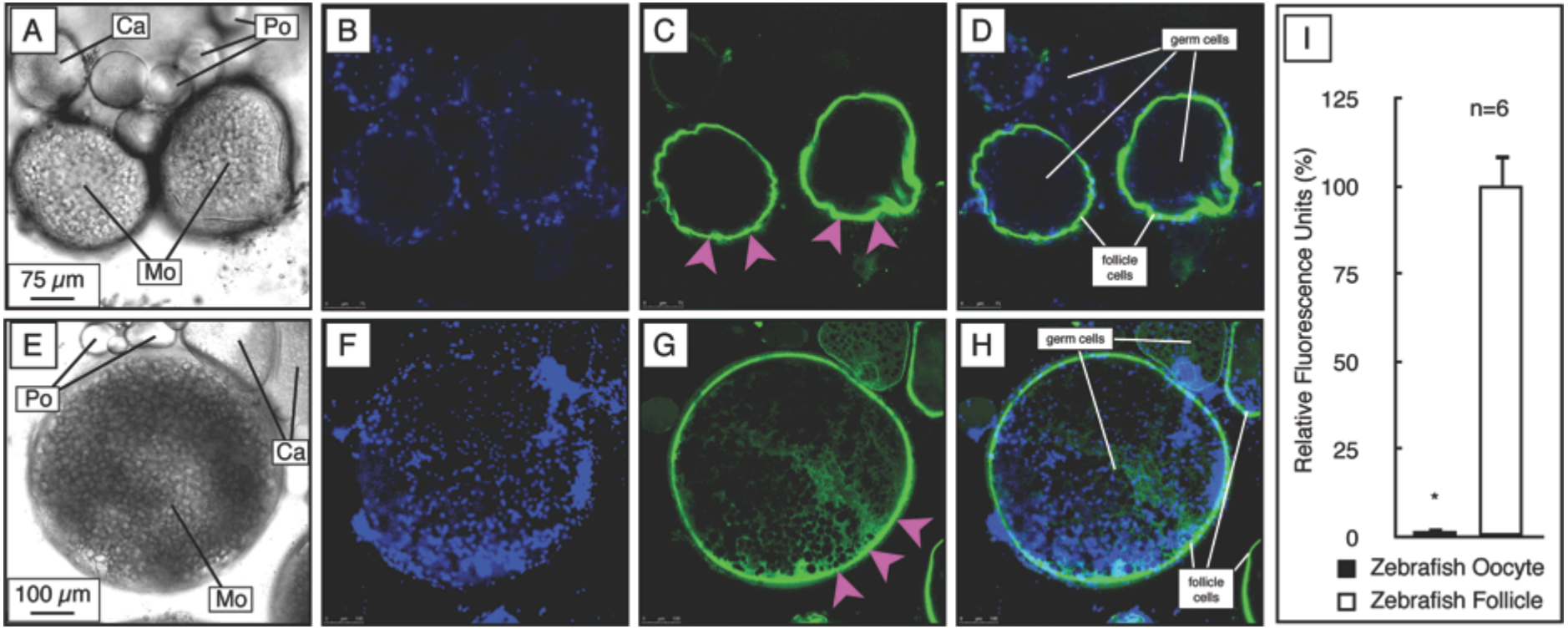

Figure 5.7. ROS accumulation in different stages of zebrafish (*Danio rerio*) oogenesis. (a,e) Bright field shows zebrafish oocytes in their different stages of development; (Po) Primary oocyte, (Ca) Cortical alveoli and (Mo) Mature oocyte stages. Scale bars are shown in the image. (b,f) DAPI stains nuclear DNA. (c,g) H_2DCF-DA reports to ROS accumulation. Magenta arrows point to the highest concentration of ROS, found in somatic follicle cells of mature oocytes. (d,h) Ovelay images of DAPI and H_2DCF-DA channels. (i) Bar graph shows the relative intensity of H_2DCF-DA fluorescence in oocytes, in comparison to follicle cells. Error bars were generated by SEM. (*) indicates $P<0.05$ (paired t-test).

In comparison with oocytes, male sperm cells seem to produce large quantities of ROS as shown in Figure 5.8. Adult male wild-type zebrafish testes were dissected and quickly probed for ROS in the same manner as described for oocytes. Sperm cells have a characteristic round head with mitochondria located at its posterior region (Figure 5.8b). Probing for ROS using H_2DCF-DA revealed that sperm mitochondria produce significant amounts of ROS, as shown in Figures 5.8e and 5.8f. This indicated that low ROS production is a sex-specific characteristic, exhibited only by the female germ line and not by male germ line cells.

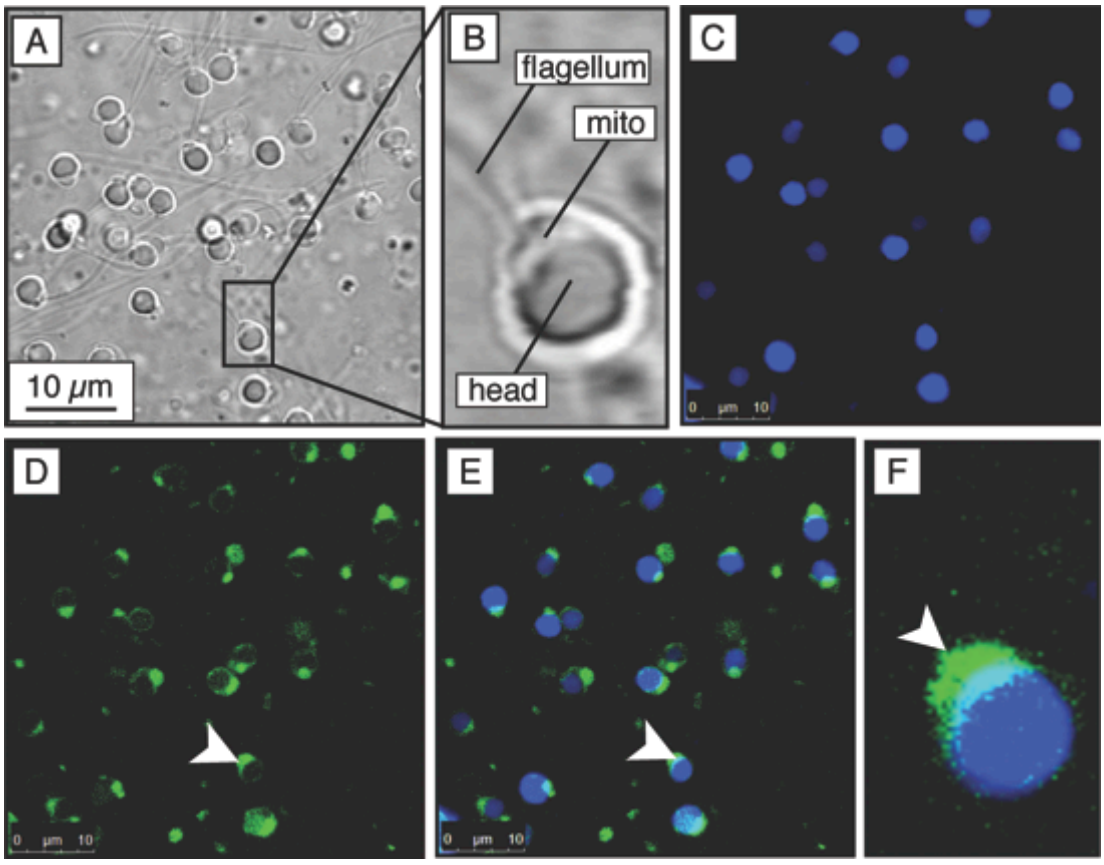

Figure 5.8. ROS production is zebrafish sperm cells. (a) Bright field shows live sperm cells released from testes. (b) Zoomed image of the sperm cell boxed in (a), indicating the location of its head, mitochondria and flagellum. (c) DAPI stains sperm nucleus, while (d) shows where ROS was found to be accumulated. (e) Overlay image of DAPI and H_2DCF-DA. (f) A zoomed image of a single sperm cell from the overlay channel. White arrows indicate sperm mitochondria.

5.3. Discussion

As discussed in Chapters 3 and 4, mitochondrial transcription and membrane potential are suppressed in the female germ line mitochondria of jellyfish, *Drosophila* and zebrafish. According to the original hypothesis (Allen, 1996), this is an in-built natural mechanism to prevent ROS production, mtDNA mutation accumulation, and inter-generation age inheritance. In accordance with this proposal, the results presented here provide another independent line of evidence concerning the ways in which oocyte mitochondria are distinct from sperm mitochondria and from mitochondria of somatic cells in those species.

Prior to this work, no study has presented results of direct measurements of ROS production specifically in oocytes, compared to other somatic cells or male gametes. Some *in vitro* fertilization (IVF) work has described methods to inhibit oxidative phosphorylation in order to reduce ROS generation, and to increase *in vitro* embryo development competence in mammals (Thompson, et al., 2000). This may be correct, but manipulation of gametes and embryos by IVF methods is known to create an unnatural environment, which often leads to oxidative stress (Guerin, et al., 2001).

In summary, this chapter presents results demonstrating that ROS content is much lower in oocytes, while not completely absent. One of the reasons why basal quantities of ROS were still detected could be the basal membrane potential found for those cells (section 4.2). If the reverse reaction of mitochondrial F_1F_0-ATP synthase and ATP hydrolysis to generate a basal membrane potential without ROS production does not occur (as proposed in section 4.3), then basal quantities of oxidative phosphorylation activity could be contributing to the basal ROS production seen in the female germ line mitochondria. Another possible explanation would be other metabolic pathways, independent of mitochondrial respiration, which could also be generating some ROS. Hydroxyl radicals can be generated via a Fenton-type mechanism in the cytoplasm, and are commonly associated with diseases involving

oxidative stress and iron accumulation (Goldstein, et al., 1993). Another source of cytoplasmic ROS can derive from NADPH oxidase reactions (Dikalov, 2011).

There is, however, some criticism of the use of H_2DCF-DA as an *in situ* cellular oxidative stress marker. Because of its hydrophilic molecular structure, it has been proposed that H_2DCF-DA cannot cross biological membranes, unless they are fenestrated (Karlsson, et al., 2010). Another criticism is that the oxidation reaction of H_2DCF-DA is strictly dependent on either Fenton-type reactions or unspecific enzymatic oxidation by cytochrome c; for it has been suggested to react with none of the ROS generated in biological processes (Karlsson, et al., 2010). Whether those claims are valid it is up to debate. If Karlsson et al. is correct, the enzyme catalase should not have any effect in the H_2DCF-DA oxidation, but that is not the case, as previously shown by (Mesquita, et al., 2010). The same applies for superoxide dismutase, which also has a clear effect in modulating H_2DCF-DA fluorescence in biological samples (Mesquita, et al., 2010).

Nevertheless, in view of such observations, *in vitro* measurements of H_2DCF-DA fluorescence (Lloyd, et al., 2012), would overcome the hindrance imposed by biological membranes. In addition, the simultaneous use of other ROS probes such as Amplex Red (Life Technologies), MitoSox (Life Technologies) or dihydroethidium (DHE) (Owusu-Ansah, et al., 2008) can be used for confirmation of the conclusion reached on the independent basis of H_2DCF-DA fluorescence.

6

Mitochondrial ultrastructure in the female germ line

In 1665, the English polymath Robert Hooke, FRS, wrote in the preface of his book entitled Micrographia (Hooke, 1665):

"…for the limits, to which our thoughts are confined, are small in respect of the vast extent of Nature it self; some parts of it are too large to be comprehended, and some too little to be perceived. And from thence it must follow, that not having a full sensation of the Object, we must be very lame and imperfect in our conceptions about it, and in all the propositions which we build upon it; hence we often take the shadow of things for the substance, small appearances for good similitudes, similitudes for definitions; and even many of those, which we think to be the most solid definitions, are rather expressions of our own misguided apprehensions than of the true nature of the things themselves."

6.1. Introduction

Since the implementation of transmission electron microscopy (TEM) in the 1950s, mitochondrial research has been boosted considerably as it allowed novel insights into the internal ultrastructure of mitochondria. These studies have revealed that mitochondria have a complex dynamic structure, and can be divided in at least six different compartments: outer membrane, inner membrane, intermembrane space, cristal membranes, intracristal space, and matrix (Logan, 2006). Their double-membrane-bounded characteristic derives from the fact that their endosymbiont ancestor was a gram-negative bacterium (Benz, 1985). The outer membrane is fenestrated and allows free passage of different hydrophilic substances with molecular weights up to 6000 Daltons (Benz, 1985). Conversely, the inner membrane is built to be a broadly impermeable insulating layer that harbours the respiratory chain and separates the intermembrane space from the mitochondrial matrix, thus preventing any proton leak that could uncouple the proton motive force from the ATP synthase (Logan, 2006). Along with the citric acid cycle enzymes and the hydrophilic subunits of the respiratory electron transport chain, the matrix also houses the mitochondrial DNA (mtDNA), in which is found to be associated with histone-free nucleoids that vary in number and content (Satoh and Kuroiwa, 1991).

Mitochondrial cristae are invaginations of the inter membrane that greatly increases the surface area, which in turn increases the effectiveness of electron transport and ATP synthesis reactions by several orders of magnitude. Thus, alterations in the number of cristae, and their morphology, can be directly associated with mitochondrial membrane potential and bioenergetic output (Zick, et al., 2009). In fact, one of the determinants of mitochondrial cristae morphology has been shown to be the dimeric and highly oligomeric state of the F_1F_0-ATP synthase. Electron cryotomography studies of intact mitochondria have revealed long rows of ATP synthase dimers associated exclusively on tightly curved cristae edges (Davies, et al., 2011). Furthermore, mutants of dimer-specific subunits, which destabilise F_1F_0-ATP

synthase supracomplexes, have shown to have a decreased mitochondrial membrane potential (Bornhovd, et al., 2006).

In animal oocytes, mitochondria are usually small spherical organelles, characterised by a pale matrix and very few small vesicular cristae, suggesting a relatively weak energetic activity and low ATP production (Van Blerkom, 2004). After fertilisation occurs, mitochondria undergo a gradual transition towards differentiation, assuming a more elongated shape, with an increase in the number of transverse cristae and denser matrix composition (Dvorak and Tesarik, 1985). These changes occur approximately at the 8-cell stage in humans, and coincide not only with the nuclear genome activation, but also with a switch in the metabolic substrates from pyruvate and lactate to glucose (Braude, et al., 1988). However, mitochondrial heterogeneity has been largely observed at these stages, suggesting that not all mitochondria to differentiate at the same rate in the early developing embryo (Jansen and de Boer, 1998).

6.2. Results

6.2.1. Mitochondrial ultrastructure in jellyfish oocytes

Figure 6.1a shows a transmission electron micrograph of a thin section of cells of a female jellyfish bell. Mitochondria (m) are seen to possess characteristic morphology, with an internal network of cristae; invaginations of the inner membrane into the mitochondrial matrix; and the sites of respiratory electron transport and ATP synthesis. Figure 6.1b shows the corresponding electron micrograph of a jellyfish oocyte; oocyte mitochondria appear slightly larger and morphologically less complex than in the bell (Figure 6.1a). Figure 6.1c shows the head of jellyfish spermatozoon in which the nucleus (n) lies adjacent to large, morphologically complex mitochondria (m) with numerous cristae. The contractile filaments of the flagellum consume ATP for the mechanochemical motor driving sperm motility and are in close proximity to the fully differentiated mitochondria, which can be assumed to function primarily as electrochemical fuel cells to supply the required ATP. In order to quantify the morphological variations seen in these tissues (Figures 6.1a-c), stereological analysis was performed in jellyfish mitochondria of bell muscle, oocyte and sperm, as shown in Figure 6.1d.

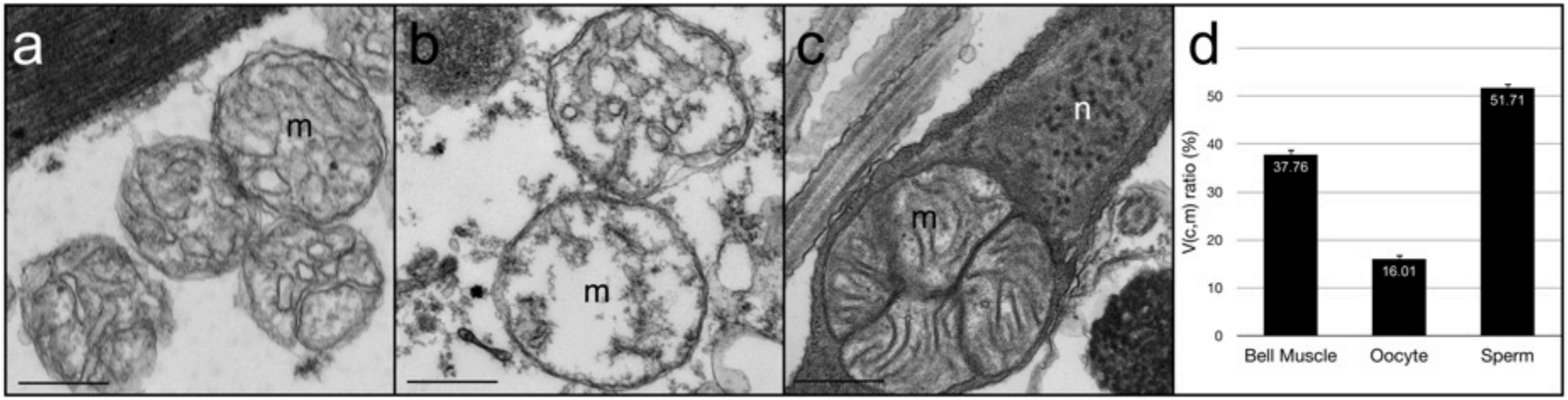

Figure 6.1. Transmission electron micrographs of *A. aurita* tissue samples. Differences in mitochondrial morphology are observed in cross-sections through (a) female bell, (b) oocyte and (c) spermatozoon. Mitochondria (m), nucleus (n). Scale bars, 500 nm. Stereological analysis of the morphological variations amongst the three samples is shown in (d). Error bars represent s.e.m, $P<0.01$.

6.2.2. Mitochondrial ultrastructure in *Drosophila melanogaster* oocytes

The ultrastructural morphology of mitochondria in different tissues of the Drosophila was performed using transmission electron micrographs of osmium-stained sections of flight muscle (Figure 6.2a), sperm (Figure 6.2b), and oocyte (Figure 6.2c). Within flight muscle of fruit fly (Figure 6.2a), the single mitochondrion in the field of view is large (approximately 2 μm in long axis) and has numerous cristae – invaginations of the mitochondrial inner membrane that together present a large total surface area for ATP synthesis by chemiosmotic energy coupling. It is also seen that the mitochondrial matrix presents a relatively high electron density, suggesting dense packing and high metabolic activity. *Drosophila* sperm mitochondria (Figure 6.2b) are unusually differentiated structures containing a paracrystalline lattice (Perotti, 1973), and therefore more difficult to compare. Conversely, *Drosophila* ooocyte (Figure 6.2c), on the same scale, contains many small mitochondria (< 0.5 μm) of much simpler morphology, present in a cytoplasm rich in ribosomes and distinct from the nucleus from which it is separated by a characteristic nuclear double membrane. The oocyte mitochondrial cristae are virtually absent and the matrix electron density is remarkably lower than the surrounding cytoplasm itself.

These results (Figure 6.2) indicate a female gamete-specific, unusual ultrastructure of oocyte mitochondria, one that is not found in diploid somatic cells or male gametes. This analysis of mitochondrial ultrastructure is indicative of a much lower mitochondrial metabolic and respiratory activity in the oocyte of *Drosophila*.

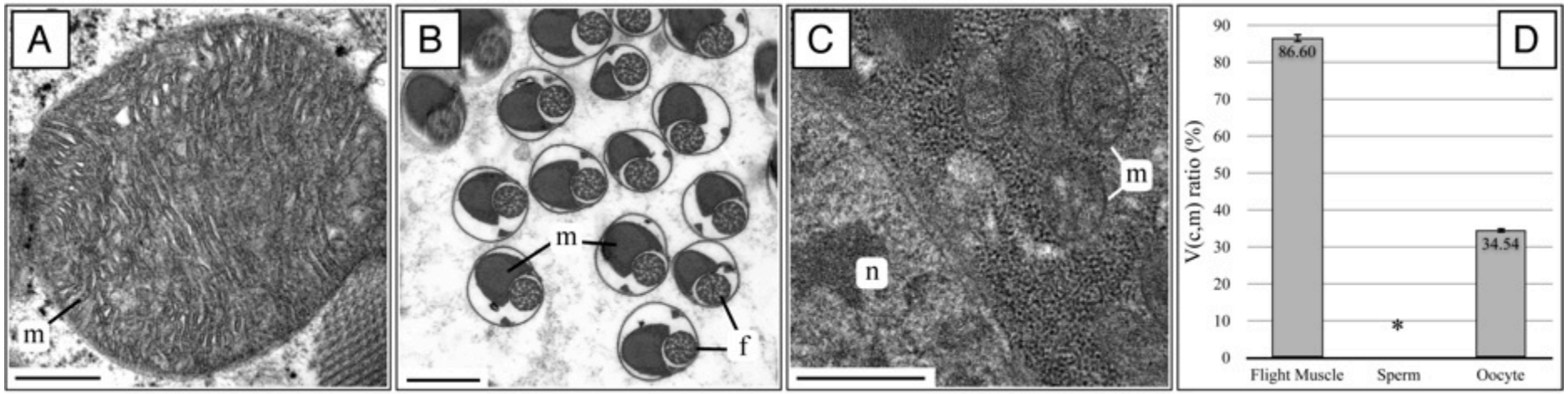

Figure 6.2. Transmission electron micrographs of *Drosophila melanogaster* (a) flight muscle, (b) sperm and (c) oocyte. Letter (m) indicates mitochondria, (n) a haploid nucleus, and (f) a flagellum. Oocyte mitochondria are seen as simpler structures, ranging from 200-500 nm, lacking cristae development and matrix electron density (c). Muscle (a) and sperm (b) mitochondria were used as a somatic and male gametic tissue control samples for normal mitochondrial development, respectively. Images were taken using 9300 X magnification. The scale bar corresponds to 500 nm. Stereological analysis of the morphological variations amongst the three samples, where V(c,m) is the ratio of crista volume to mitochondrion volume. Error bars are S.E.M, $P \leq 0.01$.

6.2.3. Mitochondrial ultrastructure in *Danio rerio* oocytes

TEM was performed to detect differences in the internal structural morphology of zebrafish oocyte mitochondria, in comparison to somatic tissues and sperm. Cardiac muscle was used as a control for active somatic mitochondria (Figure 6.3a), and sperm as a control for gametes (Figure 6.3b). Similar to what was seen for *Drosophila*, zebrafish oocyte mitochondria (Figure 6.3c) are much smaller in size, ranging from 0.3-0.6 μm in the long axis, and were also seen with a much less complex internal network of cristae. In some cases, as in the one shown in Figure 6.3c, oocyte mitochondria could even be seen completely devoid of cristae. Zebrafish sperm mitochondria (Figure 6.3b) show typical mitochondrial inner-membrane invagination and matrix density characteristic of metabolically active mitochondria.

Furthermore, stereological analysis was used to calculate the ratio of crista volume to mitochondrion volume (Figure 6.3d), and revealed that oocyte mitochondria have only 35 % of their internal volume occupied by cristae, while cardiac muscle mitochondria have in average 82 %, and sperm mitochondria 45 %. This phenotype of simpler internal complexity seen in oocyte mitochondria seems to be part of a sex-specific division of labour highly conserved throughout the animal kingdom, as seen for jellyfish (Figure 6.1) and *Drosophila melanogaster* (Figure 6.2), species spanning at least 500 million years in evolution.

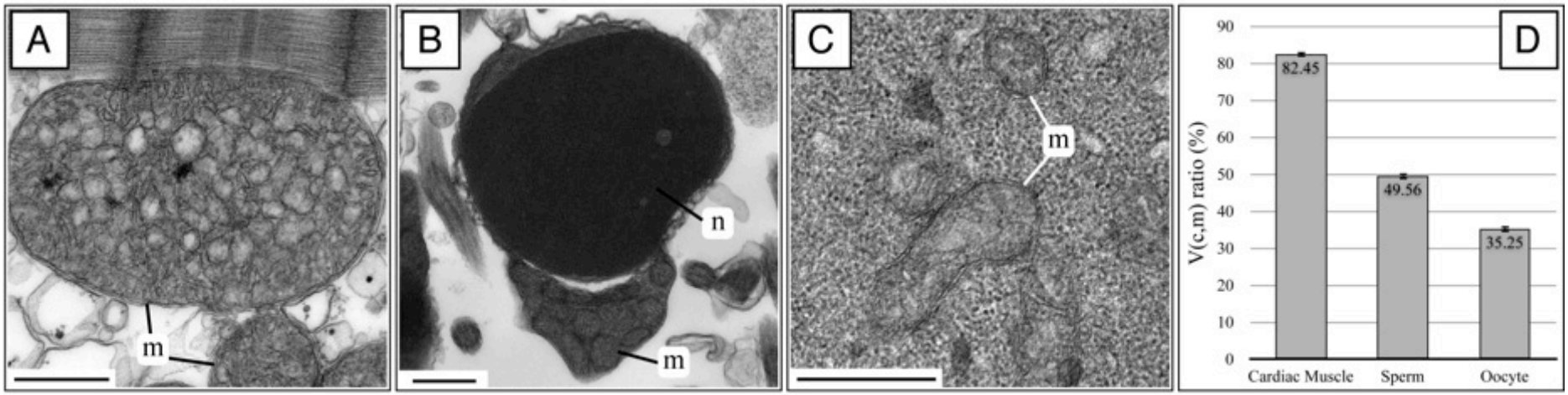

Figure 6.3. Transmission electron microscopy analysis of the zebrafish *Danio rerio* (a) cardiac muscle, (b) sperm and (c) oocyte. Letter (m) indicates mitochondria, (n) a haploid nucleus. Images were taken using 9300 X magnification. The scale bar corresponds to 500 nm. (d) Stereological analysis of the morphological variations amongst the three samples, where V(c,m) is the ratio of crista volume to mitochondrion volume. Error bars are S.E.M, $P \leq 0.01$.

6.3. Discussion

Taken together the results obtained from these three highly diverged species, it is plausible to conclude that the oocyte mitochondria remain with an undifferentiated phenotype, suggesting a low electron transport and ATP synthesis output. These results agree with the previous literature for human oocytes (Van Blerkom, 2004), as well as with the data discussed earlier in Chapters 3, 4 and 5, which show that mitochondrial transcription, membrane potential and ROS production are low in the oocyte mitochondria.

The fact that oocyte mitochondria lack a complex internal network of cristae may correlate with the lack of F_1F_0-ATP synthase in the inter membrane, which is known to be responsible for the maintenance of cristae morphology in active differentiated mitochondria (Davies, et al., 2011). Another possibility is that, F_1F_0-ATP synthase is unable to form dimers or high oligomers due to some post-translational modifications specific to oocytes, possibly at the subunits *e* and *g*, which destabilise dimeric and oligomeric F_1F_0-ATP synthase supra-complexes.

In addition, some genes that are known to be involved in the formation and maintenance of the mitochondrial cristae network could also be down regulated in the special case of the female germ cells. One example is the phosphatase and tensin homologue-induced kinase 1 (PINK1), which is a mitochondrial serine/threonine kinase. Knockout mutants encoding for PINK1 show signs of mitochondrial pathology, including signs of cristae fragmentation (Exner, et al., 2007). The optic atrophy 1 (*opa1*) gene is also a good candidate to be more down regulated in oocyte mitochondria, as *opa1* knockout mutants show loss of cristae phenotype and inter membrane vesiculation (Ishihara, et al., 2006). Another interesting gene involved in the control of mitochondrial cristae development is the *mdm33* found in *Saccharomyces cerevisiae*. Its protein product is found in the mitochondrial inter membrane, and when overexpressed, *mdm33* leads to growth arrest, aggregation of mitochondria, and generation of aberrant inner membrane structures, including

septa, inner membrane fragments, and loss of inner membrane cristae (Messerschmitt, et al., 2003).

Another interesting feature of the oocyte mitochondria that may be sometimes taken for granted is its spherical shape. Traditionally, the mitochondrion is always portrayed as a static body, ranging from 0.5-10 μm in size, but in reality, mitochondria are very dynamic organelles, capable of changing size and shape in a matter of seconds via fusion and fission (Logan, 2006). When images of taken by TEM show an elongated mitochondria, typical in most of somatic tissues, it can also be interpreted as a snap shot taken of mitochondria that are in the process of fission or fusion. Therefore, the characteristic spherical shape of the oocyte mitochondria reveals that besides not having much bioenergetic output, they could also be static, thus undergoing neither fission nor fusion. The implications of this observation could be extended to the interpretations that oocyte mitochondria are not only metabolic inactive, but also disengaged from mitochondrial biogenesis.

Recent advancements in the field of genomics have identified a large number of proteins that are directly involved in the regulation of the internal mitochondrial ultrastructure. Taken together with these results, they open a vast range of future experiments that could be performed, in order to identify specific mechanisms involved in the control of mitochondrial morphology, biogenesis and cristae development in the special case of the female germ line mitochondria. Transcription and post-translational modification analysis of these specific genes and their respective protein products would reveal what mechanisms are used by the female germ line to maintain their mitochondria round and undifferentiated. The function of these genes could also be directly relevant to maintain the mitochondrial DNA protected against respiratory activity during female germ line development and years of oocyte arrest in animals, especially that mitochondrial morphology has been shown to have a direct correlation with electron transport and membrane potential (Zick, et al., 2009).

7

General discussion

In 1860, the English naturalist Charles R. Darwin, FRS, wrote in his letter to J. S. Henslow (Burkhardt, et al., 1994):

"*There is no greater mystery in the whole world, as it seems to me, than the existence of the sexes, – more especially since the discovery of Parthenogenesis. The origination of the sexes seems beyond all speculation.*"

7.1. Mitochondria and their genomes

Mitochondria are cytoplasmic organelles of eukaryotes. Their most conspicuous role in cellular metabolism is respiration, which includes the stepwise oxidation of imported pyruvate, release of carbon dioxide, and the coupling of electron transport to synthesis of ATP in oxidative phosphorylation. In many eukaryotes, the terminal respiratory electron acceptor is oxygen. Aerobic respiration takes place in mitochondria containing a small, specialised genome and a complete apparatus of gene expression, distinct from that of the cell nucleus and cytosol.

The genomes of mitochondria contain a minute subset of genes (Anderson, et al., 1981) retained from their α-proteobacterial ancestors (Esser, et al., 2004), with other genes of prokaryotic, endosymbiont origin having been either lost, or else retained as genes now found only on chromosomes of the cell nucleus. Genes in mitochondria always encode mitochondrial proteins or RNAs (Pesole, et al., 2012), and their gene products are never exported. Nonetheless, the mitochondrial proteome is comprised of thousands of proteins (Calvo and Mootha, 2010; Rhee, et al., 2013; Smith, et al., 2012), perhaps as many as its bacterial ancestor, and the great majority are imported from the cytosol as precursors (Dudek, et al., 2013; Schmidt, et al., 2010). As a consequence, the mitochondrial inner membrane contains respiratory chain complexes with a mix of imported nuclear-encoded subunits and subunits encoded by the mitochondrial DNA and synthesised by 70S bacterial-type ribosomes in the mitochondrial matrix (Rich, 2003).

If the overwhelming majority of mitochondrial proteins are now encoded in the nucleus for synthesis in the cytoplasm, then why not all? Why has mitochondrial DNA been retained consistently in all eukaryotic branches? What is it about the genes encoded there, or their gene products, that demands a separate, dedicated, cytoplasmic genetic system in a primarily energy-transducing organelle?

7.2. Co-location for Redox Regulation (CoRR)

One hypothesis for the evolution and retention of the cytoplasmic genomes of bioenergetics organelles – both mitochondria and chloroplasts – was proposed explicitly in 1993 (Allen, 1993), and later termed "CoRR", which stands for "Co-location (of gene and gene product) for Redox Regulation (of gene expression)" (Allen, 2003; Allen, 2003).

The central proposition of the CoRR hypothesis is that a requirement for regulation of gene expression by redox state of electron carriers existed in these organelles' bacterial ancestors, and continued through the transition from free-living prokaryote to eukaryotic organelle. CoRR proposes that the genes that are subject to redox control remain in the same subcellular compartment as their gene products. The relative abundance, or stoichiometry, of these gene products is thereby rapidly and unconditionally adjusted in response to environmental changes in availability of electron sources and sinks, and to changes in energetic and metabolic supply and demand. CoRR further proposes that mitochondria and chloroplasts have retained formerly bacterial redox signalling pathways that facilitate the required regulatory coupling between electron transport and gene expression. It is predicted that genes for components of these pathways are encoded in the nucleus, since they encode part of the major class of organellar proteins and have no need, themselves, to be subject to redox regulatory control.

In fact, when the conserved patterns of genes retained in the chloroplasts of five well diverged photosynthetic lineages is represented in a Venn diagram (Figure 7.1), a process of adaptative convergence becomes clear. From all possibilities, chloroplasts have chosen to retain genes encoding core subunits of photosynthetic redox centres, and a minimum subset of ribosomes necessary to carry out in situ translation, thus chloroplasts are enables to exert gene regulatory control over its own photosynthetic rate-limiting steps (Allen, et al., 2011)

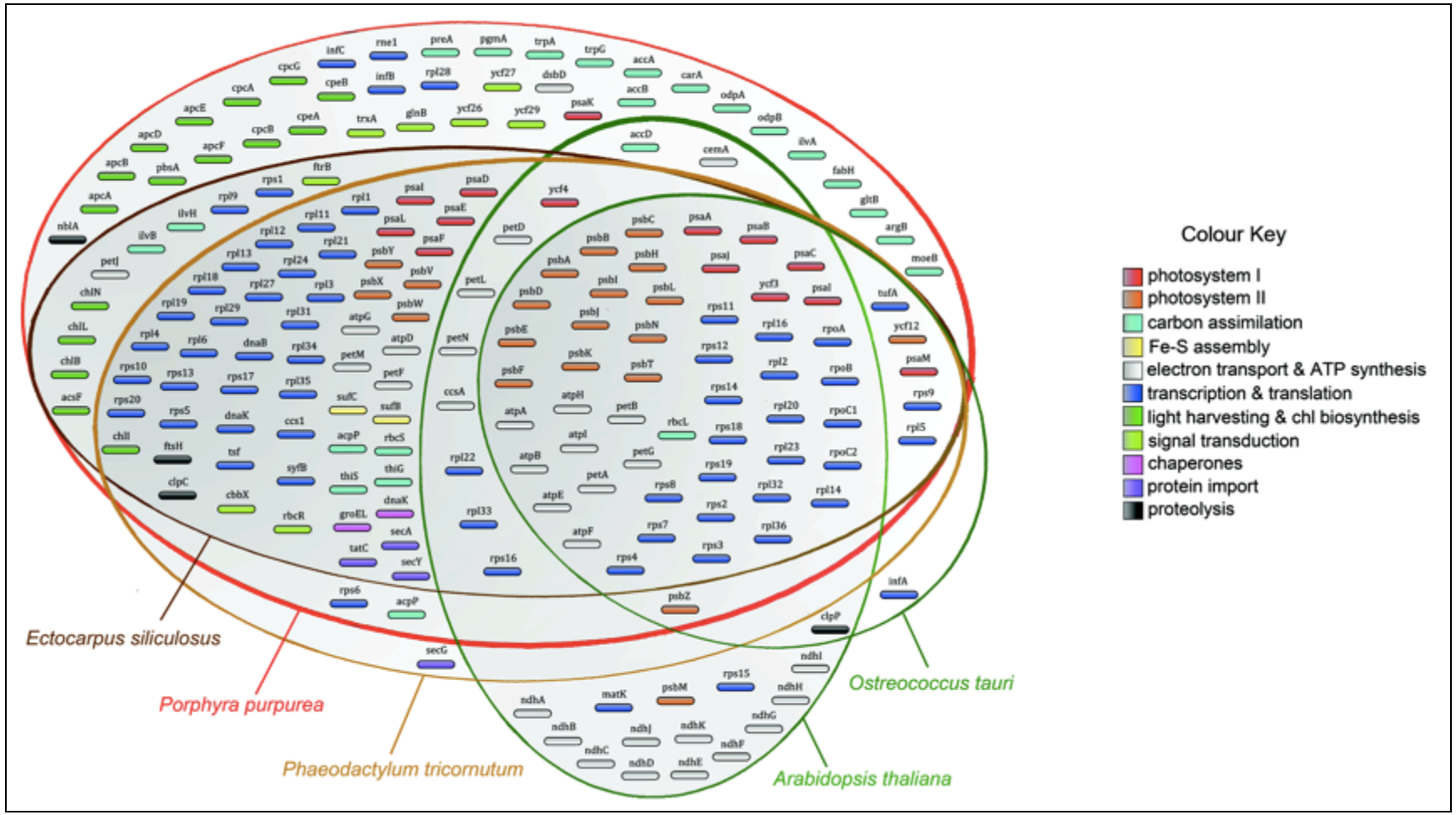

Figure 7.1. Venn diagram of gene content of the chloroplast genomes of *Arabidopsis thaliana*, the green alga *Ostreococcus tauri*, the red alga *Porphyra purpurea*, the brown macroalga *Ectocarpus siliculosus*, and the diatom *Phaelodactelum tricornutum*.

Subsequent research, specifically on chloroplast gene expression, is consistent with the CoRR hypothesis. There is evidence of favour of chloroplast CoRR in at least four independent ways: (i) chloroplast transcription is differentially regulated by redox state of a specific component of the photosynthetic electron transport chain (Pfannschmidt, et al., 1999); (ii) the effect of this regulation is to adjust the stoichiometry of photosystem I and photosystem II, restoring the redox poise of the plastoquinone pool after it has been perturbed by transient changes in light quality or by site-specific inhibition of electron transport (Allen and Pfannschmidt, 2000; Pfannschmidt, et al., 1999); (iii) a cyanobacterial redox sensor kinase, part of a two component regulatory system, is retained in chloroplasts of plants and eukaryotic algae (Puthiyaveetil, et al., 2008); (iv) this Chloroplast Sensor Kinase (CSK) is required for regulation of photosynthetic reaction centre gene transcription and is nuclear-encoded (Puthiyaveetil, et al., 2010; Puthiyaveetil, et al., 2008). Further experiments indicate that the redox transcriptional control pathway involving CSK has been partially rewired: although it still terminates with a plastid-encoded RNA polymerase, chloroplast redox signalling also governs the action of a protein kinase of eukaryotic origin (Puthiyaveetil, et al., 2013).

If the CoRR hypothesis has led to better understanding of the evolution, function and regulation of the chloroplast genome, what is the result of its application to mitochondria?

7.3. Does CoRR apply to mitochondria, too?

Evidence compatible with CoRR included effects of redox reagents and electron transport inhibitors on specific patterns of protein synthesis, probed by ^{35}S-methionine labelling both in isolated mitochondria and in isolated chloroplasts (Allen, et al., 1995). Subsequent experiments with plant mitochondria implicate respiratory complex II, which is usually alone in being a wholly nuclear encoded respiratory complex, as the site of redox control (Escobar Galvis, et al., 1998). This result is consistent with the key cofactor being ubiquinone, the mitochondrial analogue of chloroplast plastoquinone. Redox control of mitochondrial RNA synthesis has also been reported (Wilson, et al., 1996).

Independent work on mitochondria, not addressed directly to the CoRR hypothesis, nevertheless fits well with the prediction that a mitochondrial genome is required to in order exert regulatory control over respiratory electron transport. Mitochondria that have lost oxidative phosphorylation have also lost their genomes. As introduced in Section 1.1.3, examples of these relict mitochondria include mitosomes of anaerobic protists such as *Entamoeba histolytica* (Clark and Roger, 1995) and *Giardia lamblia* (Tovar, et al., 2003). Hydrogenosomes (Lindmark and Muller, 1973) carry out energy transduction, but have lost membrane-intrinsic complexes of oxidative phosphorylation. Hydrogenosomes have, accordingly, no genomes of their own (van der Giezen, 2009). Taken together, these lines of evidence suggest that retention of a mitochondrial genome may correlate with retention of aerobic oxidative phosphorylation, and that transmission of mitochondrial genomes acquires an additional constraint in fully aerobic animals and plants (Allen, et al., 2007).

A modified two-component redox regulatory systems lies at the centre of chloroplast redox control of transcription (Puthiyaveetil, et al., 2013). Mitochondrial two-component systems have been described in fungi (Mavrianos, et al., 2013) but not in animals (Wuichet, et al., 2010). A single-component redox regulator or activator can transmit redox information to regulate transcription in bacteria

(reviewed by (Allen, 1993)) and could, on its own, satisfy the requirement of CoRR. The Hydrogen Hypothesis for the origin of mitochondria and eukaryotes (Martin and Muller, 1998) envisages a metabolically and genetically flexible ancestral proteobacterium, giving rise to each of the different types of mitochondria by selective loss of function. Familiar, aerobic mitochondria of animals may therefore retain redox-signalling systems, as yet incompletely characterised.

7.4. Adjustment of respiratory chain complex stoichiometry by means of ubiquinone redox control of mitochondrial gene transcription

The chloroplast gene regulatory system relies on a bacterial-type transcriptional machinery, while many mitochondria may have lost such a system during their evolution, replacing it with a phage-type machinery (Hedtke, et al., 1997). It is plausible to suggest that mitochondria may have rewired bacterial redox regulatory components in order to maintain ubiquinone redox balance. This mechanism may include phosphorylation of a phage-type RNA polymerase integrating mitochondrial transcription factors with a mitochondrial redox sensor kinase, rather than phosphorylation of bacterial-type sigma factors, as seen for chloroplasts (Puthiyaveetil, et al., 2013).

According to this scheme envisaged in Figure 7.2, an elevated ratio of ubiquinol to ubiquinone inhibits the kinase activity of the mitochondrial ubiquinone redox sensor, and, as a consequence, mitochondrial transcription factors become free to bind to mitochondrial DNA and to facilitate the transcription of genes for core subunits of its primary and secondary electron acceptors, complexes III and IV, as shown in Figure 7.2a. Increased electron flow out, and decreased electron flow in then restore optimal redox state of the quinone pool, where ubiquinone (oxidised) and ubiquinol (reduced) are present in a ratio of one-to-one. Conversely, a decreased ubiquinol-to-ubiquinone ratio activates the kinase domain of the proposed mitochondrial redox sensor. This in turn phosphorylates the mitochondrial transcription factors, leading to transcriptional suppression of the Complex III and IV core subunit genes. The proposed effect of this redox control, indicated in Figure 7.2b, will be decrease electron flow out, and increase electron flow in, again restoring ubiquinone redox poise.

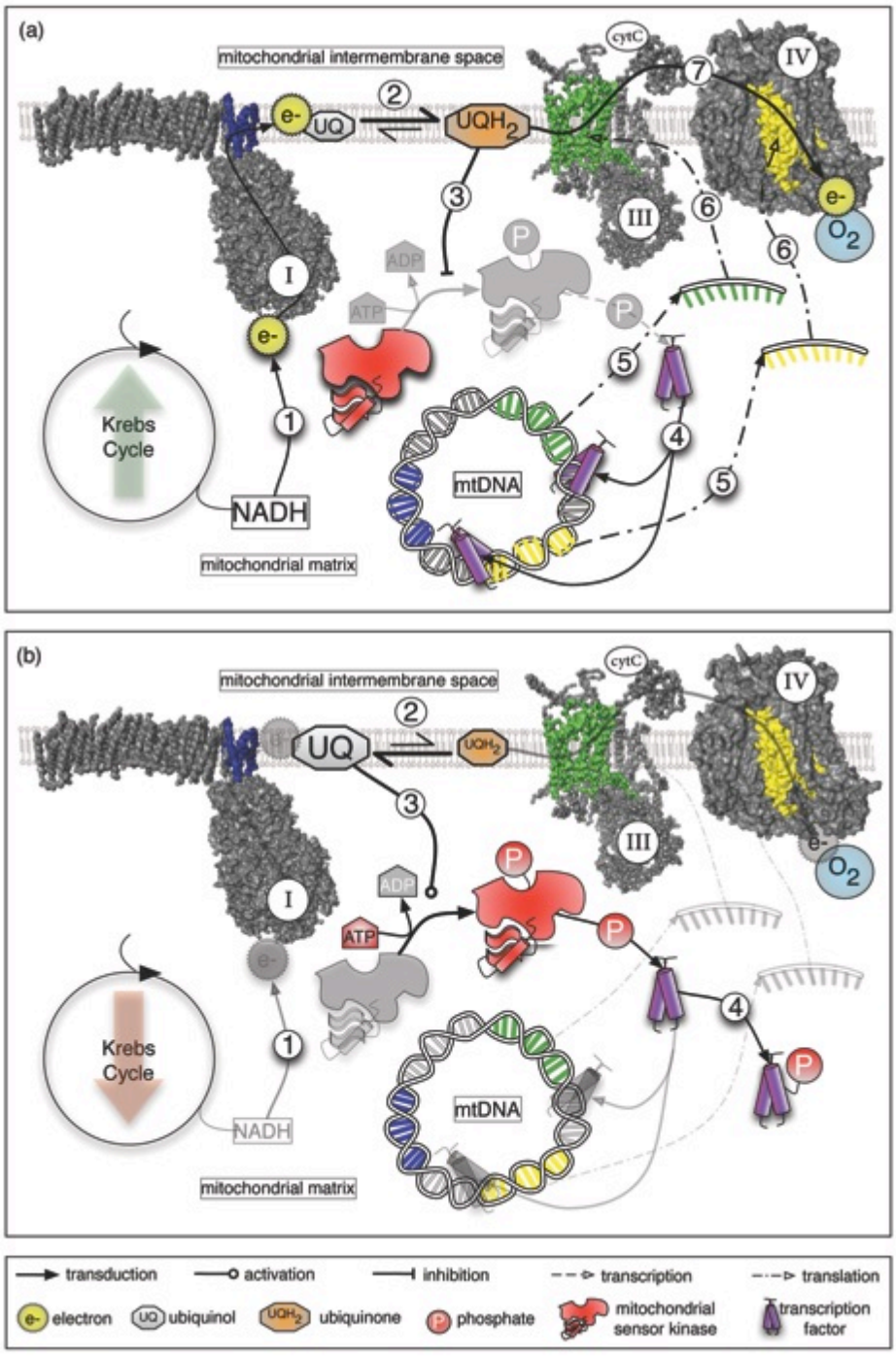

Figure 7.2. Proposed regulatory control of mitochondrial transcription by the redox state of the ubiquinol/ubiquione pool. (a) An elevated NADH output from the Krebs cycle (1) increases the ubiquinol to ubiquinone ratio (2) and inhibits the kinase activity of the mitochondrial redox sensor (3), which allows transcription factors to bind to mtDNA (4) to promote transcription (5) and translation (6) of *cob* and *cox1* genes, subsequently increasing the electron flow out toward molecular oxygen as the final acceptor (7). (b) Conversely, if NADH supply is diminished (1), the ubiquinone pool becomes more oxidised (2). This activates the kinase domain of the mitochondrial redox sensor (3), which in turn phosphorylates the transcription factor, precluding its binding to mtDNA and upregulation of transcription.

7.5. Requirement for redox poise

A self-regulatory mechanism of the kind shown in Figure 7.2 may operate even in anaerobic mitochondria, where it may serve to increase efficiency by matching respiratory complex stoichiometry to the kinetics of electron flow. In plants and microorganisms with branched respiratory chains, a prolonged redox imbalance may also send signals to initiate *de novo* synthesis of additional or alternative electron donors or acceptors. However, maintaining ubiquinone redox poise may be especially important in the presence of molecular oxygen. The reason for this is that ubisemiquinone, a transient intermediate in complexes I and III, itself carries an unpaired electron, and reacts readily with molecular oxygen to yield the superoxide anion radical (Chance, et al., 1975; Chen, et al., 2003). Superoxide is indiscriminately reactive and mutagenic, and is also a precursor of related and cytotoxic reactive oxygen species (ROS). Therefore a precisely balanced respiratory chain will help to minimise destructive effects of oxygen chemistry, not only on the chain itself, but also on the genes for its core components. Thus, if there is a benefit in having a mitochondrial genome, there is also, potentially, a serious, and long-lasting cost (Allen, 1996) in the form of accumulated mis-coding of mitochondrial apoproteins that are correspondingly more likely to catalyse destructive single-electron transfers to oxygen. This "vicious circle" of DNA-damage giving protein-damage giving more DNA-damage (Ernster, 1994), highlights what otherwise amounts to a "design flaw" in aerobic, eukaryotic cells. Without the benefit of a redox autoregulatory system of the kind depicted in Figure 7.2, mitochondria are about the worst imaginable location in the cell to store a genetic system. In the absence of CoRR, all formerly endosymbiont genes would be expected to have re-located the metabolically and electrochemically inert compartment represented by the cell nucleus.

7.6. The mitochondrial theory of ageing

The mitochondrial theory of ageing (Harman, 1972) states that superoxide anions ($O_2^{\bullet -}$), produced by single-electron transfers in the mitochondrial respiratory chain, react with the DNA retained in the mitochondrial matrix, causing deleterious rearrangements that accumulate over time (Ames, et al., 1993; Ames, et al., 1995). There is debate on whether ROS are the direct cause of the decline of mitochondrial function seen in ageing (Afanas'ev, 2010; Jacobs, 2003), while recent studies are certainly consistent with the theory. Superoxide production increases with age of brain tissue in a senescence-accelerated mouse line, in rats, and in pigeons (Sasaki, et al., 2008). Oxidative damage and apoptosis occur during ageing in the brain, oculus and kidney of mice and are accompanied by increased $O_2^{\bullet -}$ production. Comparatively lower $O_2^{\bullet -}$ concentrations are found in cardiac and muscle tissues (Miyazawa, et al., 2009). Pressure-induced arterial $O_2^{\bullet -}$ increases with age (Jacobson, et al., 2007), and endogenous extracellular superoxide dismutase may play a role in protection against endothelial dysfunction during ageing in mice (Lund, et al., 2009). In humans, there is an age-related increase of lipoperoxides, and decrease of glutathione peroxidase activity in blood plasma (Mendoza-Nunez, et al., 2007). In addition, endothelial oxidative stress develops with ageing in healthy men and is related to impaired vascular endothelial function (Donato, et al., 2007; Mendoza-Nunez, et al., 2007).

7.7. Why is acquired mitochondrial mutation not inherited?

If ROS-induced mitochondrial DNA mutation drives the ageing process, then it becomes necessary to explain the failure of ageing to be inherited, along with the damaged mitochondrial DNA. One proposal is that maternally transmitted mitochondria form part of a continuous, quiescent lineage of cytoplasmic genetic templates, inactive in transcription and oxidative phosphorylation, and are thus exempt from the ageing process (Allen, 1996). Figure 7.3a illustrates this hypothesis of a self-contained life cycle of template mitochondria in females. Figure 7.3b diagrammatically distinguishes mitochondrial from nuclear "germ plasm", a distinction unknown at the time of Weismann's proposed "central dogma" of continuity of the germ plasm (Weismann, 1889), and of the non-inheritance of somatically acquired characters.

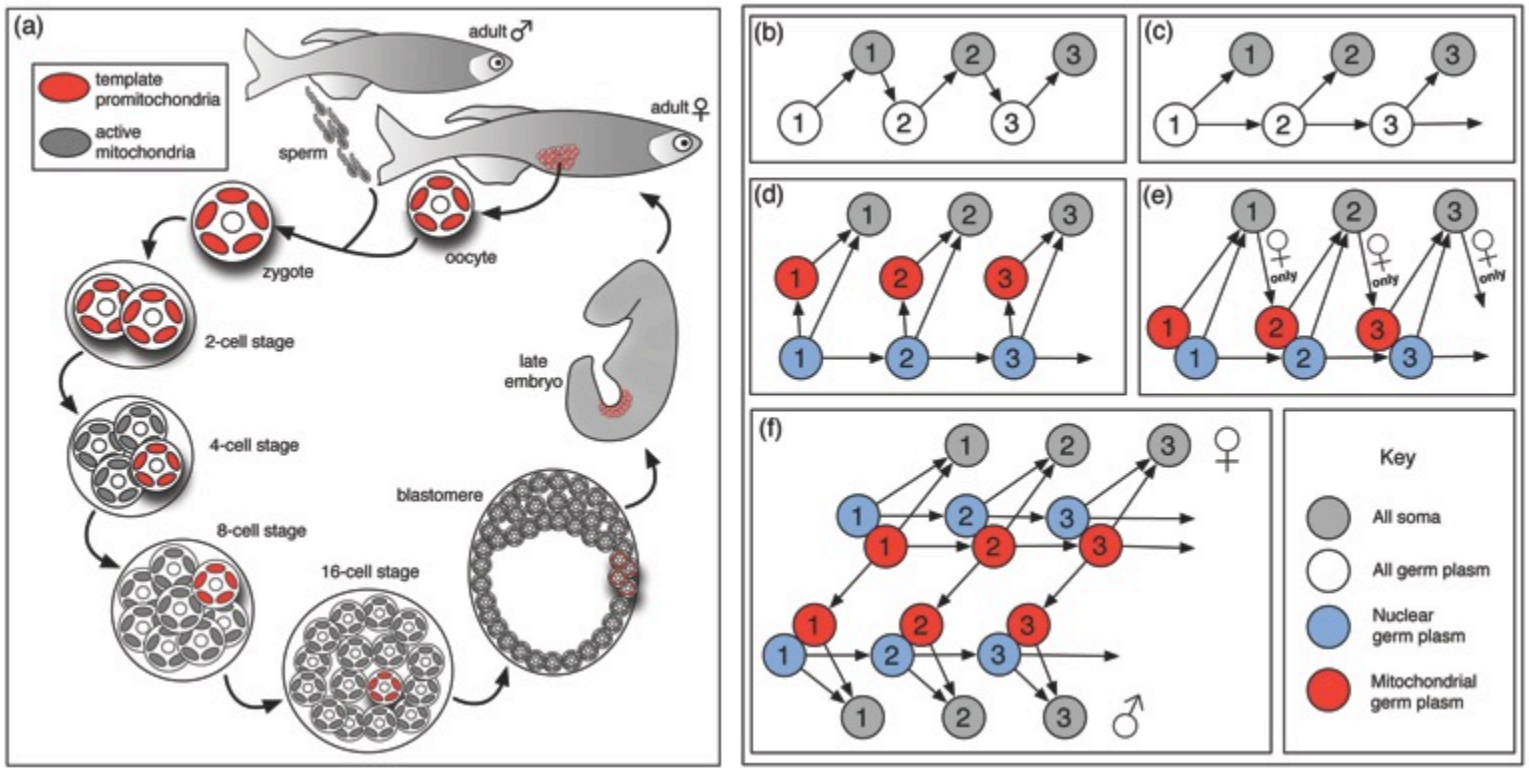

Figure 7.3. Continuity of the mitochondrial germ plasm. (a) Illustration of maintenance of a distinct population of promitochondria (red) as quiescent genetic templates in the female germ line and throughout development. (b) The pre-Weismann view, associated with Lamarck, of inheritance of characters acquired by the soma (grey) and transmitted in the germ plasm (white). (c) Weismann's hypothesis of the continuity of the germ plasm from which the soma derives. (d) A view, now discounted, of mitochondria (red) as a component of the soma, and arising from the nucleus (blue). (e) A current consensus view, raising the problem of maternal inheritance of mitochondrial DNA, which should transmit age-related mitochondrial mutation if mitochondrial germ plasm (red) derives from soma (grey). (f) Mitochondrial division of labour between male and female, preserving the Weismann barrier for mitochondrial as well as for nuclear inheritance.

7.8. Evidence for quiescent, template mitochondria in the female germ-line

Using evolutionarily widely separated model species, the jellyfish, *Aurelia aurita*, the fruitfly, *Drosophila melanogaster*, and the zebrafish, *Danio rerio*, it was observed a decreased transcription of three mitochondrial genes for respiratory chain proteins in ovary, as compared to testis and to somatic tissues, as reported in Chapter 3. The three genes chosen for comparison were *nad1*, *cob*, and *cox1*, encoding core protein subunits of the major respiratory complexes I, III and IV, as shown in the scheme in Figure 3.1.

Confocal light microscopy was also performed to examine and compare mitochondrial membrane potential (Chapter 4), and reactive oxygen species (Chapter 5). In all three sample animal species, it was seen that oocyte mitochondria stand out from those in neighbouring cells, and from those of sperm and somatic cells, in their low intensity Mitotracker red fluorescence. A corresponding pattern of oocyte mitochondrial quiescence was observed for ROS as reported by 2',7'-dihydrodichlorofluorescein diacetate (H_2DCF-DA) – oocytes from all three species produce fewer ROS when compared to other tissues.

Using transmission electron microscopy (TEM) of tissue sections, Chapter 6 reports that oocyte mitochondria are distinct in size and ultrastructure. Oocytes carry small mitochondria that are simple in internal membrane topology, having few cristae – the invaginations of the mitochondrial inner membrane that carry the respiratory chain complexes and ATP synthase of oxidative phosphorylation. Structurally simple mitochondria have been reported to accompany suppressed respiratory and complex IV activity in oocytes of the amphibian, *Xenopus laevis* (Kogo, et al., 2011). By analogy with proplastids of plant and algal cells, the term promitochondria (Allen, 1996) is used to describe these organelles.

7.9. An evolutionary perspective on mitochondria, separate sexes, and the female germ line

In summary, this work leads to the conclusion that oocyte mitochondria serve as quiescent genetic templates, and are bioenergetically dormant. Fertilization requires sperm motility, and sperm mitochondria are active in ATP synthesis. Elimination of the incoming, paternal, sperm mitochondria from the zygote underlies mitochondrial maternal inheritance (Al Rawi, et al., 2011; Deluca and O'farrell, 2013). This selective elimination, leaving only quiescent promitochondria provided by the oocyte, may explain the non-inheritance of mitochondrial DNA mutations accumulated with ageing. By permitting a new population of mitochondria to arise from a fresh and uncorrupted template in each generation, continuity of a mitochondrial germ plasm may be preserved (Figure 7.3a,f).

It remains to be demonstrated that a continuous line of cells, forming the female germ line, is maintained from oocyte, through each generation, to oocyte, as illustrated in Figure 7.3a and 7.3f. Reversion of functional and differentiated mitochondria to promitochondria at any stage of development will falsify the hypothesis by (Allen, 1996). Experimental evidence for discontinuity of promitochondria in the female germ line, and to understand the mechanisms controlling mitochondrial bioenergetics in the female germ line is a priority for future research.

7.10. Future directions

One of the important directions indicated by the work presented in this thesis is to elucidate the cellular mechanisms involved in the control of mitochondrial bioenergetics during oocyte and embryonic development, and to understand how repression of respiration in oocytes can prevent the accumulation of mitochondrial DNA (mtDNA) mutations that can lead to mitochondrial and age-related diseases in the offspring. Furthermore, it would be interesting to dissect the signalling transduction pathways connecting the control of respiratory metabolism with cell division cycle mechanisms in animal oocytes.

MtDNA mutations accumulate with time in energetically active somatic cell mitochondria, and are thought to be the main cause of ageing and inherited mitochondrial disorders (Wallace, 2010). Consequently, mitochondria that have not been kept suppressed in primordial oocytes throughout decades of a mother's lifetime can accumulate hazardous quantities of mtDNA mutations. These accumulated oocyte mtDNA mutations acquired during early and adult life cannot be selected against by genetic mechanisms, such as the mitochondrial genetic bottleneck (Wai, et al., 2008) or purifying selection mechanisms (Fan, et al., 2008; Stewart, et al., 2008). This could be one of the main reasons why inherited mitochondrial and age-related diseases still occur at relatively frequent rates in the population. Therefore, it is of immense importance to understand the mechanisms suppressing the respiratory activity of oocyte mitochondria, especially in the case of primary oocytes of humans, which can remain arrested for years, or even decades.

One strong candidate for the control of oocyte mitochondrial activity is the cyclic adenosine monophosphate (cAMP)-dependent pathway. It is well known that cAMP is the universal inhibitor of cell cycle and oocyte maturation (Bravo, et al., 1978; Meijer, et al., 1989), and cyclic nucleotide phosphodiesterases (PDEs) are responsible for cAMP hydrolysis and resumption of meiosis during oocyte maturation (Masciarelli, et al., 2004). In the mitochondrial proteome, a complete cAMP-signaling system is present, including a soluble adenylyl cyclase (sAC), a

cAMP-dependent kinase (PKA) and a mitochondrial PDE (Acin-Perez, et al., 2011). When the concentrations of cAMP are high, active PKA regulates respiration by phosphorylating subunits of the mitochondrial electron transport chain (ETC) complexes (Acin-Perez, et al., 2009). Furthermore, PKA has also been shown to phosphorylate the mitochondrial transcription factor A (TFAM), thus impairing its ability to bind DNA and to activate mitochondrial transcription (Lu, et al., 2013). Additionally, yeast mutants lacking a mitochondrial PDE show a 4-fold decreased respiratory activity in the presence of exogenous cAMP, when compared to no cAMP added, or to wild-type strains (Leadsham and Gourlay, 2010).

Another protein strongly implicated in control of mitochondrial DNA metabolism is the mitochondrial single-stranded DNA-binding protein (mtSSB). MtSSB is abundant in mitochondria of primary and mature oocytes of *Xenopus laevis* (Ghrir, et al., 1991), *Drosophila melanogaster* (Maier, et al., 2001) and *Caenorhabditis elegans* (Sugimoto, et al., 2008). Interestingly, mtSSB seems to have a dual function in animal mitochondria: at the same time that it promotes mtDNA replication by enhancing mitochondrial DNA polymerase activity (Farr, et al., 1999), it also inhibits transcription of mtDNA (Barat-Gueride, et al., 1989). Furthermore, binding activity of mtSSB to mtDNA appears to be regulated by phosphorylation and mediated by CDK-CKS complexes (Radulovic, et al., 2010), which are in turn regulated by cellular concentrations of cAMP (Desdouets, et al., 1995).

Taken together, these lines of evidence lead to the big question: While cytosolic cAMP effectors maintain primary oocytes arrested in the presence of cAMP, do intra-mitochondrial cAMP effectors work synergistically to maintain their mitochondria quiescent, and to protect the precious maternal mtDNA against years of exposure to the damaging by-products of respiration? In turn, does cAMP breakdown restore mitochondrial activity to provide the energy necessary for resumption of meiosis in initiation of embryo development? To date, no work has attempted to establish such vital connections between cell division cycle, oocyte maturation and mitochondrial bioenergetics, and these are the questions my future research plans aim to provide the answers for.

Appendix

Disclosures

- In Chapter 1, parts of the text have been published in the book chapter (de Paula, et al., 2012);

- Some parts of Chapter 3, 4, 5 and 6 involving work with jellyfish have been published in (de Paula, et al., 2013). Other parts involving work with *Drosophila* and zebrafish have been submitted for publication and are currently under review (de Paula, et al., 2013);

- The Venn diagram of the genes in chloroplast, Figure 7.1, is the result of my original work, published in (Prihoda, et al., 2012), and also similarly in (Allen, et al., 2011);

- Some parts discussed in Chapter 7, including Figures 7.2 and 7.3 are published in (Allen and de Paula, 2013);

- The work presented in Chapter 6 was done by myself and Dr. Gema Vizcay-Barrena, at the Centre for Ultrastructural Imaging (CUI), King's College London. Dr. Vizcay-Barrena assisted me with sample preparation, heavy-metal staining and performed the stereological analysis;

- Mr. Ahmed-Noor A. Agip assisted with tissue dissection for the *Drosophila* and zebrafish work.

Bibliographical references

Bibliographical references

Acin-Perez, R., *et al.* (2011) A phosphodiesterase 2A isoform localized to mitochondria regulates respiration, *J Biol Chem*, **286**, 30423-30432.

Acin-Perez, R., *et al.* (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation, *Cell Metab*, **9**, 265-276.

Acton, B.M., *et al.* (2004) Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development, *Mol Hum Reprod*, **10**, 23-32.

Adrian, G.S., *et al.* (1986) Sequences required for delivery and localization of the ADP/ATP translocator to the mitochondrial inner membrane, *Mol Cell Biol*, **6**, 626-634.

Afanas'ev, I. (2010) Signaling and Damaging Functions of Free Radicals in Aging-Free Radical Theory, Hormesis, and TOR, *Aging Dis*, **1**, 75-88.

Al Rawi, S., *et al.* (2011) Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission, *Science*, **334**, 1144-1147.

Albertini, D.F. and Anderson, E. (1974) The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions, *J Cell Biol*, **63**, 234-250.

Alfonso-Prieto, M., *et al.* (2009) The Molecular Mechanism of the Catalase Reaction, *J Am Chem Soc*, **131**, 11751-11761.

Allen, C.A., Hakansson, G. and Allen, J.F. (1995) Redox Conditions Specify the Proteins Synthesized by Isolated- Chloroplasts and Mitochondria, *Redox Rep.*, **1**, 119-123.

Allen, C.A., van der Giezen, M. and Allen, J.F. (2007) Origin, function and transmission of mitochondria. In Martin, W. and Muller, M. (eds), *Origins of Mitochondria and Hydrogenosomes*. Springer-Verlag, Berlin Heidelberg, pp. 39-56.

Allen, J.F. (1993) Control of Gene-Expression by Redox Potential and the Requirement for Chloroplast and Mitochondrial Genomes, *Journal of Theoretical Biology*, **165**, 609-631.

Allen, J.F. (1993) Redox Control of Transcription - Sensors, Response Regulators, Activators and Repressors, *FEBS Lett.*, **332**, 203-207.

Allen, J.F. (1996) Separate Sexes and the Mitochondrial Theory of Ageing, *Journal of Theorical Biology*, **180**, 135-140.

Allen, J.F. (2003) The function of genomes in bioenergetic organelles, *Philosophical Transactions of the Royal Society B: Biological Sciences*, **358**, 19-38.

Allen, J.F. (2003) Why chloroplasts and mitochondria contain genomes, *Comp Funct Genomics*, **4**, 31-36.

Allen, J.F., Alexciev, K. and Hakansson, G. (1995) Photosynthesis. Regulation by redox signalling, *Curr Biol*, **5**, 869-872.

Allen, J.F., *et al.* (2011) A structural phylogenetic map for chloroplast photosynthesis, *Trends in plant science*, **16**, 645-655.

Allen, J.F. and de Paula, W.B.M. (2013) Mitochondrial genome function and maternal inheritance, *Biochem. Soc. Trans.*, "**in press**".

Allen, J.F. and Pfannschmidt, T. (2000) Balancing the two photosystems: photosynthetic electron transfer governs transcription of reaction centre genes in chloroplasts, *Philos Trans R Soc Lond B Biol Sci*, **355**, 1351-1359.

Allen, J.F. and Raven, J.A. (1996) Free-Radical-Induced Mutation vs Redox Regulation: Costs and Benefits of Genes in Organelles, *Journal of Molecular Evolution*, **42**, 482-492.

Altmann, R. (1890) Die Elementarorganismen und ihre Beziehunger zu den Zellen., *Leipzig*.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging, *P Natl Acad Sci USA*, **90**, 7915-7922.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1995) Mitochondrial decay in aging, *Biochim Biophys Acta*, **1271**, 165-170.

Anderson, E. and Albertini, D.F. (1976) Gap junctions between the oocyte and companion follicle cells in the mammalian ovary, *J Cell Biol*, **71**, 680-686.

Anderson, S., *et al.* (1981) Sequence and organization of the human mitochondrial genome, *Nature*, **290**, 457-465.

Antelman, J., *et al.* (2008) Expression of Mitochondrial Transcription Factor A (TFAM) During Porcine Gametogenesis and Preimplantation Embryo Development, *Journal of Cellular Physiology*, **217**, 529-543.

Ashburner, M. and Novitski, E. (1976) *The Genetics and biology of Drosophila*. Academic Press, London ; New York.

Atteia, A., *et al.* (2009) A proteomic survey of Chlamydomonas reinhardtii mitochondria sheds new light on the metabolic plasticity of the organelle and on the nature of the alpha-proteobacterial mitochondrial ancestor, *Molecular Biology and Evolution*, **26**, 1533–1548.

Ayala, F.J. (1998) Is sex better? Parasites say "no", *Proc Natl Acad Sci U S A*, **95**, 3346-3348.

Bai, J.X., *et al.* (1999) Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury, *Journal of Biological Chemistry*, **274**, 26217-26224.

Barat-Gueride, M., Dufresne, C. and Rickwood, D. (1989) Effect of DNA conformation on the transcription of mitochondrial DNA, *European journal of biochemistry / FEBS*, **183**, 297-302.

Bastock, R. and St Johnston, D. (2008) Drosophila oogenesis, *Current biology*, **18**, R1082-1087.

Bekker, M., *et al.* (2010) The ArcBA two-component system of Escherichia coli is regulated by the redox state of both the ubiquinone and the menaquinone pool, *J Bacteriol*, **192**, 746-754.

Bell, G. (1978) The evolution of anisogamy, *Journal of Theoretical Biology*, **73**, 247-270.

Benton, M.J. and Donoghue, P.C.J. (2006) Paleontological Evidence to Date the Tree of Life, *Molecular Biology and Evolution*, **24**, 26-53.

Benz, R. (1985) Porin from bacterial and mitochondrial outer membranes, *CRC critical reviews in biochemistry*, **19**, 145-190.

Berlett, B.S. and Stadtman, E.R. (1997) Protein oxidation in aging, disease, and oxidative stress, *The Journal of biological chemistry*, **272**, 20313-20316.

Birky, C. (2009) Giardia Sex? Yes, but how and how much?, *Trends in Parasitology*, **26**, 70-74.

Bornhovd, C., *et al.* (2006) Mitochondrial membrane potential is dependent on the oligomeric state of F1F0-ATP synthase supracomplexes, *J Biol Chem*, **281**, 13990-13998.

Bradley, P.J., *et al.* (1997) Targeting and translocation of proteins into the hydrogenosome of the protist Trichomonas: similarities with mitochondrial protein import, *Embo J*, **16**, 3484-3493.

Braude, P., Bolton, V. and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development, *Nature*, **332**, 459-461.

Bravo, R., *et al.* (1978) Amphibian oocyte maturation and protein synthesis: related inhibition by cyclic AMP, theophylline, and papaverine, *Proc Natl Acad Sci U S A*, **75**, 1242-1246.

Brinkmann, H., *et al.* (2005) An empirical assessment of long-branch attraction artefacts in deep eukaryotic phylogenomics, *Systems Biology*, **54**, 743-757.

Broughton, R.E., Milam, J.E. and Roe, B.A. (2001) The complete sequence of the zebrafish (Danio rerio) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial DNA, *Genome Res*, **11**, 1958-1967.

Bui, E.T., Bradley, P.J. and Johnson, P.J. (1996) A common evolutionary origin for mitochondria and hydrogenosomes, *P Natl Acad Sci USA*, **93**, 9651-9656.

Burkhardt, F., *et al.* (1994) *A Calendar of the Correspondence of Charles Darwin, 1821-1882*. Cambridge University Press.

Butterfield, N.J. (1997) Plankton ecology and the Proterozoic-Phanerozoic transition, *Paleobiology*, **23**, 247-262.

Butterfield, N.J. (2000) *Bangiomorpha pubescens* n. gen., n. sp.: implications for the evolution of sex, multicellularity, and the Mesoproterozoic/Neoproterozoic radiation of eukaryotes, *Paleobiology*, **26**, 386-404.

Calvo, S.E. and Mootha, V.K. (2010) The mitochondrial proteome and human disease, *Annu Rev Genomics Hum Genet*, **11**, 25-44.

Cao, L., *et al.* (2007) The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells, *Nature genetics*, **39**, 386-390.

Cao, L., *et al.* (2009) New evidence confirms that the mitochondrial bottleneck is generated without reduction of mitochondrial DNA content in early primordial germ cells of mice, *PLoS Genet*, **5**, e1000756.

Cartwright, P., *et al.* (2007) Exceptionally Preserved Jellyfishes from the Middle Cambrian, *PLoS One*, **2**.

Cavalier-Smith, T. (1983) A 6 kingdom classification and a unified phylogeny. In: Schwemmler W, Schenk HEA (eds) Endocytobiology II., *De Gruyter, Berlin*, pp 1027–1034.

Cerkasovova, A., *et al.* (1976) Circular DNA and cardiolipin in hydrogenosomes, microbody-like organelles of trichomonads, *Folia Parasitol (Praha)*, **23**, 33-37.

Chance, B., Saronio, C. and Leigh, J.S., Jr. (1975) Functional intermediates in reaction of cytochrome oxidase with oxygen, *P Natl Acad Sci USA*, **72**, 1635-1640.

Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide Metabolism in Mammalian Organs, *Physiological Reviews*, **59**, 527-605.

Chappel, S. (2013) The Role of Mitochondria from Mature Oocyte to Viable Blastocyst, *Obstetrics and Gynecology International*, **2013**.

Chen, J., Tauer, G. and Huang, Y. (2002) Paternal chloroplast inheritance patterns in pine hybrids detected with trnL-trnF intergenic region polymorphism, *Theor Appl Genet*, **104**, 1307-1311.

Chen, L.B. (1988) Mitochondrial membrane potential in living cells, *Annu Rev Cell Biol*, **4**, 155-181.

Chen, Q., *et al.* (2003) Production of reactive oxygen species by mitochondria - Central role of complex III, *Journal of Biological Chemistry*, **278**, 36027-36031.

Choksi, K.B., *et al.* (2007) Lower levels of F2-isoprostanes in serum and livers of long-lived Ames dwarf mice, *Biochem Biophys Res Commun*, **364**, 761-764.

Cipriani, G., Testolin, R. and Morgante, M. (1995) Paternal inheritance of plastids in interspecific hybrids of the genus Actinidia revealed by PCR-amplification of chloroplast DNA fragments, *Mol Gen Genet*, **247**, 693-697.

Clark, C., *et al.* (2007) Structure and content of the *Entamoeba histolytica* genome, *Adv Parasitology*, **65**, 51-190.

Clark, C. and Roger, A. (1995) Direct evidence for secondary loss of mitochondria in *Entamoeba histolytica*, *Proceedings of the National Academy of Sciences*, **92**, 6518–6521.

Claros, M.G., *et al.* (1995) Limitations to in vivo import of hydrophobic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome b, *Eur. J. Biochem.*, **228**, 762-771.

Collins, A.G., *et al.* (2006) Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models, *Syst Biol*, **55**, 97-115.

Condon, R.H., *et al.* (2012) Questioning the Rise of Gelatinous Zooplankton in the World's Oceans, *Bioscience*, **62**, 160-169.

Cooke, M.S., *et al.* (2003) Oxidative DNA damage: mechanisms, mutation, and disease, *Faseb J*, **17**, 1195-1214.

Cosmides, L.M. and Tooby, J. (1981) Cytoplasmic inheritance and intragenomic conflict, *Journal of Theoretical Biology*, **89**, 83-129.

Cox, R.T. and Spradling, A.C. (2003) A Balbiani body and the fusome mediate mitochondrial inheritance during Drosophila oogenesis, *Development*, **130**, 1579-1590.

Daley, D.O., Clifton, R. and Whelan, J. (2002) Intracellular gene transfer: reduced hydrophobicity facilitates gene transfer for subunit 2 of cytochrome c oxidase, *Proc Natl Acad Sci U S A*, **99**, 10510-10515.

Dalton, C.M. (2011) Mitochondrial dynamics during mouse oocyte maturation. *School of Life and Medical Sciences*. UCL (University College London), London, UK, pp. 189p.

Davies, K.M., *et al.* (2011) Macromolecular organization of ATP synthase and complex I in whole mitochondria, *Proc Natl Acad Sci U S A*, **108**, 14121-14126.

Dawson, M.N. and Martin, L.E. (2001) Geographic variation and ecological adaptation in *Aurelia* (Scyphozoa, Semaeostomeae): some implications from molecular phylogenetics, *Hydrobiologia*, **451**, 259-273.

de Paula, W.B.M., *et al.* (2013) Function, ultrastructure, and transcription in mitochondria of female and male gametes: the germ line in maternal transmission of mitochondrial DNA, **Under review.**

de Paula, W.B.M., Allen, J.F. and van der Giezen, M. (2012) Mitochondria, Hydrogenosomes and Mitosomes in Relation to the CoRR Hypothesis for Genome Function and Evolution, *in: Organelle genetics. C.E. Bullerwell (ed.) Springer-Verlag Berlin Heidelberg*, **105-119**.

de Paula, W.B.M., *et al.* (2013) Energy, ageing, fidelity and sex: oocyte mitochondrial DNA as a protected genetic template, *Philos Trans R Soc Lond B Biol Sci*, **368**, 20120263.

Deluca, Steven Z. and O'farrell, Patrick H. (2013) Barriers to Male Transmission of Mitochondrial DNA in Sperm Development, *Developmental Cell*, **22**, 660-668.

Desdouets, C., *et al.* (1995) Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM, *Mol Cell Biol*, **15**, 3301-3309.

Dikalov, S. (2011) Cross talk between mitochondria and NADPH oxidases, *Free Radical Biology and Medicine*, **51**, 1289-1301.

Dobbs, S.T. and Moore, A.L. (1987) Ohmicity, Proticity and Electron Flux in Mung Bean Mitochondria. In Moore, A.L. and Beechey, R.B. (eds), *Plant Mitochondria:Structural, Functional, and Physiological Aspects*. Plenum Press, New York, pp. 414.

Donato, A.J., *et al.* (2007) Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB, *Circ Res*, **100**, 1659-1666.

Doolittle, W.F. (1998) You are what you eat: a gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes, *Trends Genet*, **14**, 307-311.

Dudek, J., Rehling, P. and van der Laan, M. (2013) Mitochondrial protein import: common principles and physiological networks, *Biochim Biophys Acta*, **1833**, 274-285.

Dumollard, R. (2004) Sperm-triggered [Ca2+] oscillations and Ca2+ homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production, *Development*, **131**, 3057-3067.

Dumollard, R., *et al.* (2006) Regulation of redox metabolism in the mouse oocyte and embryo, *Development*, **134**, 455-465.

Dvorak, M. and Tesarik, J. (1985) Differentiation of mitochondria in the human preimplantation embryo grown *in vitro*., *Scripta Medica (Brno)*, **3**, 161-169.

Ellis, J.R., Bentley, K.E. and McCauley, D.E. (2008) Detection of rare paternal chloroplast inheritance in controlled crosses of the endangered sunflower Helianthus verticillatus, *Heredity*, **100**, 574-580.

Ernster, L. (1994) Keilin Memorial Lecture. The merger of bioenergetics and molecular biology, *Biochemical Society transactions*, **22**, 253-265.

Escobar Galvis, M.L., Allen, J.F. and Hakansson, G. (1998) Protein synthesis by isolated pea mitochondria is dependent on the activity of respiratory complex II, *Curr Genet*, **33**, 320-329.

Esser, C., *et al.* (2004) A genome phylogeny for mitochondria among alpha-proteobacteria and a predominantly eubacterial ancestry of yeast nuclear genes, *Mol Biol Evol*, **21**, 1643-1660.

Esser, C., Martin, W. and Dagan, T. (2007) The origin of mitochondria in light of a fluid prokaryotic chromosome model, *Biol Lett*, **3**, 180-184.

Exner, N., *et al.* (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **27**, 12413-12418.

Extavour, C.G. and Akam, M. (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation, *Development*, **130**, 5869-5884.

Falkenberg, M., *et al.* (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA, *Nature Genetics*, **31**, 289-294.

Fan, W., *et al.* (2008) A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations, *Science*, **319**, 958-962.

Farr, C.L., *et al.* (2004) Physiological and biochemical defects in functional interactions of mitochondrial DNA polymerase and DNA-binding mutants of single-stranded DNA-binding protein, *The Journal of biological chemistry*, **279**, 17047-17053.

Farr, C.L., Wang, Y. and Kaguni, L.S. (1999) Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis, *The Journal of biological chemistry*, **274**, 14779-14785.

Fernandes, R., *et al.* (2012) NLRP5 mediates mitochondrial function in mouse oocytes and embryos, *Biology of Reproduction*, **86**, 138, 131-110.

Fillingame, R.H. (1990) Molecular mechanics of ATP synthesis by F1F0-type H+-transporting ATP synthases, *Bacteria*, **12**, 345.

Fisher, R.A. (1930) *The Genetical Theory of Natural Selection*. The Clarendon Press, Oxford.

Fortey, R.A. (1998) *Life : an unauthorised biography : a natural history of the first four thousand million years of life on Earth*. Flamingo, Hammersmith, London.

Gechev, T.S., *et al.* (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death, *Bioessays*, **28**, 1091-1101.

Geissler, A., *et al.* (2000) Membrane potential-driven protein import into mitochondria. The sorting sequence of cytochrome b(2) modulates the deltapsi-dependence of translocation of the matrix-targeting sequence, *Mol Biol Cell*, **11**, 3977-3991.

Ghrir, R., *et al.* (1991) Primary structure of the two variants of Xenopus laevis mtSSB, a mitochondrial DNA binding protein, *Arch Biochem Biophys*, **291**, 395-400.

Ghrir, R., *et al.* (1991) Primary structure of the two variants of Xenopus laevis mtSSB, a mitochondrial DNA binding protein, *Arch Biochem Biophys*, **291**, 395-400.

Goldstein, S., Meyerstein, D. and Czapski, G. (1993) The Fenton Reagents, *Free Radical Biology and Medicine*, **15**, 435-445.

Grace, S.C. (1990) Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria, *Life Sci*, **47**, 1875-1886.

Gray, A. (1887) *The elements of botany for beginners and for schools*. American Book Co., New York,.

Gray, M.W. (1998) Rickettsia, typhus and the mitochondrial connection, *Nature*, **396**, 109-110.

Gray, M.W. and Doolittle, W.F. (1982) Has the endosymbiont hypothesis been proven?, *Microbiol Rev*, **46**, 1-42.

Greenleaf, A.L., Kelly, J.L. and Lehman, I.R. (1986) Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome, *P Natl Acad Sci USA*, **83**, 3391-3394.

Guerin, P., El Mouatassim, S. and Menezo, Y. (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings, *Human reproduction update*, **7**, 175-189.

Guixe, V., *et al.* (1994) Glycolysis Is Operative in Amphibian Oocytes, *FEBS Lett.*, **343**, 219-222.

Hackstein, J.H., *et al.* (1999) Hydrogenosomes: eukaryotic adaptations to anaerobic environments, *Trends Microbiol*, **7**, 441-447.

Hadjivasiliou, Z., *et al.* (2012) Selection for mitonuclear co-adaptation could favour the evolution of two sexes, *Proceedings of the Royal Society B: Biological Sciences*, **279**, 1865-1872.

Hansen, A.K., *et al.* (2007) Paternal, maternal, and biparental inheritance of the chloroplast genome in Passiflora (Passifloraceae): implications for phylogenetic studies, *Am J Bot*, **94**, 42-46.

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry, *J Gerontol*, **11**, 298-300.

Harman, D. (1972) The biologic clock: the mitochondria?, *J Am Geriatr Soc*, **20**, 145-147.

Harman, D. (1992) Free radical theory of aging, *Mutat Res*, **275**, 257-266.

Harman, D. and Harman, H. (2003) "I thought, thought, thought for four months in vain and suddenly the idea came"--an interview with Denham and Helen Harman. Interview by K. Kitani and G.O. Ivy, *Biogerontology*, **4**, 401-412.

Hedtke, B., Borner, T. and Weihe, A. (1997) Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis, *Science*, **277**, 809-811.

Hellemans, J., *et al.* (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, *Genome Biol*, **8**, R19.

Henze, K. and Martin, W. (2003) Essence of mitochondria, *Nature*, **426**, 127-128.

Hirst, A.G. and Lucas, C.H. (1998) Salinity influences body weight quantification in the scyphomedusa *Aurelia aurita*: important implications for body weight determination in gelatinous zooplankton, *Mar Ecol Prog Ser*, **165**, 259-269.

Holland, H.D. (2006) The oxygenation of the atmosphere and oceans, *Philos T R Soc B*, **361**, 903-915.

Hooke, R. (1665) *Micrographia: or, Some physiological descriptions of minute bodies made by magnifying glasses. With observations and inquiries thereupon*. Printed by J. Martyn and J. Allestry - Royal Society, London.

Horner, D.S., *et al.* (1996) Molecular data suggest an early acquisition of the mitochondrion endosymbiont, *Proc Biol Sci*, **263**, 1053-1059.

Howard, C.V. and Reed, M.G. (2010) *Unbiased Stereology: Three-dimensional measurement in Microscopy.* . QTP Publications, Liverpool, UK.

Howe, D.G., *et al.* (2013) ZFIN, the Zebrafish Model Organism Database: increased support for mutants and transgenics, *Nucleic Acids Res*, **41**, D854-860.

Hsieh, R.H., *et al.* (2004) Decreased expression of mitochondrial genes in human unfertilized oocytes and arrested embryos, *Fertility and sterility*, **81 Suppl 1**, 912-918.

Hurst, L. and Hamilton, W. (1992) Cytoplasmic Fusion and the Nature of Sexes, *Proceedings of the Royal Society B: Biological Sciences*, **247**, 189-194.

Ibrahim, I.M. (2009) Characterizing chloroplast sensor kinase, *Bioscience Horizons*, **2**, 191-196.

Igosheva, N., *et al.* (2010) Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes, *PLoS One*, **5**, e10074.

Ishihara, N., *et al.* (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1, *Embo J*, **25**, 2966-2977.

Jacobs, H.T. (2003) The mitochondrial theory of aging: dead or alive?, *Aging Cell*, **2**, 11-17.

Jacobson, A., *et al.* (2007) Aging enhances pressure-induced arterial superoxide formation, *Am J Physiol Heart Circ Physiol*, **293**, H1344-1350.

Jacobson, M.D. (1996) Reactive oxygen species and programmed cell death, *Trends in Biochemical Sciences*, **21**, 83-86.

Jang, S.H. and Jaehning, J.A. (1991) The yeast mitochondrial RNA polymerase specificity factor, MTF1, is similar to bacterial sigma factors, *The Journal of biological chemistry*, **266**, 22671-22677.

Jansen, R.P. and de Boer, K. (1998) The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate, *Molecular and cellular endocrinology*, **145**, 81-88.

Jenuth, J.P., *et al.* (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA, *Nature genetics*, **14**, 146-151.

Jo, S.H., *et al.* (2001) Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial NADP+-dependent isocitrate dehydrogenase, *J Biol Chem*, **276**, 16168-16176.

Johnson, L.V., *et al.* (1981) Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy, *The Journal of cell biology*, **88**, 526-535.

Johnson, M.T., *et al.* (2007) Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo, *Biol Reprod*, **77**, 2-8.

Kameyama, Y., *et al.* (2007) Characterization of mitochondrial replication and transcription control during rat early development in vivo and in vitro, *Reproduction*, **133**, 423-432.

Kanevski, I. and Maliga, P. (1994) Relocation of the plastid rbcL gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts, *Proc Natl Acad Sci U S A*, **91**, 1969-1973.

Karlsson, M., *et al.* (2010) What does the commonly used DCF test for oxidative stress really show?, *Biochem J*, **428**, 183-190.

Keller, A. (2007) Drosophila melanogaster's history as a human commensal, *Current biology : CB*, **17**, R77-81.

Knowlton, N. (1974) A note on the evolution of gamete dimorphism, *Journal of Theoretical Biology*, **46**, 283-285.

Koç, K.D., Y., A. and Yüce, R. (2008) Ovary Maturatıon Stages and Histological Investigation of Ovary of the Zebrafish, *Brazilian Archives of Biology and Technology*, **51**, 513-522.

Kocabas, A.M., *et al.* (2006) The transcriptome of human oocytes, *P Natl Acad Sci USA*, **103**, 14027-14032.

Kogo, N., *et al.* (2011) Germ-line mitochondria exhibit suppressed respiratory activity to support their accurate transmission to the next generation, *Dev Biol*, **349**, 462-469.

Konstantinov, Y.M., Tarasenko, V.I. and Rogozin, I.B. (2001) Redox modulation of the activity of DNA topoisomerase I from carrot (Daucus carota) mitochondria, *Doklady Biochemistry and Biophysics*, **377**, 263-265.

Krisher, R.L. and Bavister, B.D. (1999) Enhanced glycolysis after maturation of bovine oocytes in vitro is associated with increased developmental competence, *Mol Reprod Dev*, **53**, 19-26.

Krisher, R.L. and Herrick, J.R. (2013) Oocyte Metabolism and Its Relationship to Developmental Competence. In Krisher, R.L. (ed), *Oocyte Physiology and Development in Domestic Animals*. Wiley-Blackwell, pp. 248p.

Kujoth, G.C., *et al.* (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging, *Science*, **309**, 481-484.

Kurland, C.G. and Andersson, S.G. (2000) Origin and evolution of the mitochondrial proteome, *Microbiol Mol Biol Rev*, **64**, 786-820.

Kussmaul, L. and Hirst, J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria, *P Natl Acad Sci USA*, **103**, 7607-7612.

LaJeunesse, D.R., *et al.* (2004) Three new Drosophila markers of intracellular membranes, *Biotechniques*, **36**, 784-788, 790.

Lane, N. (2011) Mitonuclear match: Optimizing fitness and fertility over generations drives ageing within generations, *Bioessays*, **33**, 860-869.

Lane, N., Allen, J.F. and Martin, W. (2010) How did LUCA make a living? Chemiosmosis in the origin of life, *BioEssays : news and reviews in molecular, cellular and developmental biology*, **32**, 271-280.

Lane, N. and Martin, W. (2010) The energetics of genome complexity, *Nature*, **467**, 929-934.

Lang, B.F., *et al.* (1997) An ancestral mitochondrial DNA resembling a eubacterial genome in miniature, *Nature*, **387**, 493-497.

Larsson, N.G., *et al.* (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice, *Nature Genetics*, **18**, 231-236.

Leadsham, J.E. and Gourlay, C.W. (2010) cAMP/PKA signaling balances respiratory activity with mitochondria dependent apoptosis via transcriptional regulation, *BMC cell biology*, **11**, 92.

Lee, H.Y., *et al.* (2010) Targeted Expression of Catalase to Mitochondria Prevents Age-Associated Reductions in Mitochondrial Function and Insulin Resistance, *Cell Metab*, **12**, 668-674.

León-Avila, G. and Tovar, J. (2004) Mitosomes of *Entamoeba histolytica* are abundant mitochondrion-related remnant organelles that lack a detectable organellar genome, *Microbiology*, **150**, 1245-1250.

Li, Y., *et al.* (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase, *Nature genetics*, **11**, 376-381.

Lill, R. and Mühlenhoff, U. (2005) Iron-sulfur-protein biogenesis in eukaryotes, *Trends Biochem Sci*, **30**, 133-141.

Lindmark, D.G. and Muller, M. (1973) Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate Tritrichomonas foetus, and its role in pyruvate metabolism, *The Journal of biological chemistry*, **248**, 7724-7728.

Lloyd, D. (2004) 'Anaerobic protists': some misconceptions and confusions, *Microbiology*, **150**, 1115-1116.

Lloyd, D. (2006) Hydrogen sulfide: clandestine microbial messenger?, *Trends Microbiol*, **14**, 456-462.

Lloyd, D., *et al.* (2012) What yeast and cardiomyocytes share: ultradian oscillatory redox mechanisms of cellular coherence and survival, *Integrative biology : quantitative biosciences from nano to macro*, **4**, 65-74.

Loftus, B., *et al.* (2005) The genome of the protist parasite Entamoeba histolytica, *Nature*, **433**, 865-868.

Logan, D.C. (2006) The mitochondrial compartment, *Journal of Experimental Botany*, **57**, 1225-1243.

Lu, B., *et al.* (2013) Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease, *Molecular cell*, **49**, 121-132.

Lucas, C.H. (2001) Reproduction and life history strategies of the common jellyfish, *Aurelia aurita*, in relation to its ambient environment, *Hydrobiologia*, **451**, 229-246.

Luck, D.J.L. and Reich, E. (1964) DNA in Mitochondria of Neurospora crassa, *Proc.Natl.Acad.Sci. U.S.A.*, **52**, 931-938.

Lund, D.D., *et al.* (2009) Protective effect of extracellular superoxide dismutase on endothelial function during aging, *Am J Physiol Heart Circ Physiol*, **296**, H1920-1925.

Madsen, J.D. and Waller, D.M. (1983) A Note on the Evolution of Gamete Dimorphism in Algae, *The American Naturalist*, **121**, 443-447.

Mai, Z., *et al.* (1999) Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("crypton") in the microaerophilic protozoan parasite *Entamoeba histolytica*, *Molecular Cell Biology*, **19**, 2198–2205.

Maier, D., *et al.* (2001) Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in Drosophila melanogaster, *Mol Biol Cell*, **12**, 821-830.

Maier, D., *et al.* (2001) Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in Drosophila melanogaster, *Mol Biol Cell*, **12**, 821-830.

Margulis, L. (1970) *Origin of Eukaryotic Cells*. Yale University Press, New Haven.

Margulis, L. (1981) *Symbiosis in cell evolution*. W.H. Freeman, New York.

Martin, W. (2007) Eukaryote and mitochondrial origins: two sides of the same coin and too much ado about oxygen. In: Falkowski P, Knoll A (eds) Primary producers of the sea. Academic Press, New York, 55-73.

Martin, W., *et al.* (2001) An overview of endosymbiotic models for the origins of eukaryotes, their ATP-producing organelles (mitochondria and hydrogenosomes), and their heterotrophic lifestyle, *Biol Chem*, **382**, 1521-1539.

Martin, W. and Kowallik, K. (1999) Annotated English translation of Mereschkowsky's 1905 paper 'Uber Natur und Ursprung der Chromatophoren im Pflanzenreiche', *European Journal of Phycology*, **34**, 287-295.

Martin, W. and Muller, M. (1998) The hydrogen hypothesis for the first eukaryote, *Nature*, **392**, 37-41.

Martin, W. and Müller, M. (2007) Origin of Mitochondria and Hydrogenosomes, *7 in color, Hardcover*, **XVIII**, 306p.

Martin, W. and Russell, M.J. (2003) On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells, *Philos Trans R Soc Lond B Biol Sci*, **358**, 59-83; discussion 83-55.

Martin, W. and Russell, M.J. (2007) On the origin of biochemistry at an alkaline hydrothermal vent, *Philos Trans R Soc Lond B Biol Sci*, **362**, 1887-1925.

Martin, W., Somerville, C. and Goer, S. (1992) Molecular Phylogenies of Plastid Origins and Algal Evolution, *Journal of Molecular Evolution*, **35**, 385-404.

Masciarelli, S., *et al.* (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility, *The Journal of clinical investigation*, **114**, 196-205.

Matova, N. and Cooley, L. (2001) Comparative aspects of animal oogenesis, *Dev Biol*, **231**, 291-320.

Mavrianos, J., *et al.* (2013) Mitochondrial two-component signaling systems in Candida albicans, *Eukaryotic cell*, **12**, 913-922.

May-Panloup, P., *et al.* (2005) Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors, *Reprod Biol Endocrinol*, **3**, 65.

Mayden, R.L., *et al.* (2007) Phylogenetic relationships of Danio within the order Cypriniformes: a framework for comparative and evolutionary studies of a model species, *J Exp Zool B Mol Dev Evol*, **308**, 642-654.

Maynard Smith, J. (1982) *Evolution and the theory of games*. Cambridge University Press, Cambridge ; New York.

McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein), *The Journal of biological chemistry*, **244**, 6049-6055.

Mehrabian, Z., *et al.* (2005) Regulation of mitochondrial gene expression by energy demand in neural cells, *J Neurochem*, **93**, 850-860.

Meijer, L., *et al.* (1989) Starfish oocyte maturation: evidence for a cyclic AMP-dependent inhibitory pathway, *Dev Biol*, **133**, 58-66.

Melov, S., *et al.* (1998) A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase, *Nature genetics*, **18**, 159-163.

Mendoza-Nunez, V.M., *et al.* (2007) Aging-related oxidative stress in healthy humans, *Tohoku J Exp Med*, **213**, 261-268.

Mereschkowsky, C.S. (1905) Uber natur and ursprung der chromatophoren im pflanzenreiche, *Biol.Zentr.*, **25**, 593-604.

Mesquita, A., *et al.* (2010) Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity, *Proc Natl Acad Sci U S A*, **107**, 15123-15128.

Messerschmitt, M., *et al.* (2003) The inner membrane protein Mdm33 controls mitochondrial morphology in yeast, *J Cell Biol*, **160**, 553-564.

Mignotte, F., Tourte, M. and Mounolou, J.C. (1987) Segregation of mitochondria in the cytoplasm of Xenopus vitellogenic oocytes, *Biol Cell*, **60**, 97-102.

Millet, C.O., *et al.* (2013) Mitochondria-derived organelles in the diplomonad fish parasite Spironucleus vortens, *Exp Parasitol*.

Minczuk, M., *et al.* (2011) TEFM (c17orf42) is necessary for transcription of human mtDNA, *Nucleic Acids Res*, **39**, 4284-4299.

Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism, *Nature*, **191**, 144-148.

Mitchell, P. (1978) David Keilin's Respiratory Chain Concept and its Chemiosmotic Consequences, *Nobel Lecture*, **Chemistry**.

Mitchell, P. (1979) Keilins Respiratory-Chain Concept and Its Chemiosmotic Consequences, *Science*, **206**, 1148-1159.

Mitchell, P.D., Skulachev, V.P. and Hinkle, P.C. (1981) *Chemiosmotic proton circuits in biological membranes : in honor of Peter Mitchell*. Addison-Wesley, Advanced Book Program/World Science Division, Reading, Mass.

Miyazawa, M., *et al.* (2009) The role of mitochondrial superoxide anion (O2(-)) on physiological aging in C57BL/6J mice, *J Radiat Res*, **50**, 73-83.

Morandini, A.C. and Da Silveira, F.L. (2001) Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae). Gametogenesis, egg release, embryonic development, and gastrulation, *Sci. Mar.*, **65**, 139-149.

Morrison, H., *et al.* (2007) Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*, *Science*, **317**, 1921-1926.

Muller, M. (1993) The hydrogenosome, *J Gen Microbiol*, **139**, 2879-2889.

Murthy, M.R.N., *et al.* (1981) Structure of Beef-Liver Catalase, *J Mol Biol*, **152**, 465-&.

Nagai, S., *et al.* (2004) Oocyte mitochondria: strategies to improve embryogenesis, *Hum Cell*, **17**, 195-201.

Naqui, A., Chance, B. and Cadenas, E. (1986) Reactive oxygen intermediates in biochemistry, *Annu Rev Biochem*, **55**, 137-166.

Nass, M.M. and Nass, S. (1963) Intramitochondrial Fibers with DNA Characteristics. I. Fixation and Electron Staining Reactions, *J Cell Biol*, **19**, 593-611.

Ohnishi, S.T., *et al.* (2010) New insights into the superoxide generation sites in bovine heart NADH-ubiquinone oxidoreductase (Complex I) The significance of protein-associated ubiquinone and the dynamic shifting of generation sites between semiflavin and semiquinone radicals, *Bba-Bioenergetics*, **1797**, 1901-1909.

Ojimi, M.C. and Hidaka, M. (2010) Comparison of telomere length among different life cycle stages of the jellyfish *Cassiopea andromeda*, *Mar. Biol.*, **157**, 2279-2287.

Omori, S., *et al.* (2007) Complete nucleotide sequences of the mitochondrial genomes of two avian malaria protozoa, Plasmodium gallinaceum and Plasmodium juxtanucleare, *Parasitol Res*, **100**, 661-664.

Owusu-Ansah, E., Yavari, A. and Banerjee, U. (2008) A protocol for in vivo detection of reactive oxygen species, *Nature Protocol Exchange*.

Paget, M.S. and Buttner, M.J. (2003) Thiol-based regulatory switches, *Annu Rev Genet*, **37**, 91-121.

Park, C.B. and Larsson, N.G. (2011) Mitochondrial DNA mutations in disease and aging, *Journal of Cell Biology*, **193**, 809-818.

Parker, G.A., Baker, R.R. and Smith, V.G.F. (1972) The origin and evolution of gamete dimorphism and the male-female phenomenon, *Journal of Theoretical Biology*, **36**, 529-553.

Parkes, T.L., *et al.* (1998) Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons, *Nature Genetics*, **19**, 171-174.

Pendergrass, W., Wolf, N. and Poot, M. (2004) Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues, *Cytometry A*, **61**, 162-169.

Perez-Brocal, V. and Clark, C.G. (2008) Analysis of two genomes from the mitochondrion-like organelle of the intestinal parasite Blastocystis: complete sequences, gene content, and genome organization, *Mol Biol Evol*, **25**, 2475-2482.

Perotti, M.E. (1973) The mitochondrial derivative of the spermatozoon of Drosophila before and after fertilization, *Journal of ultrastructure research*, **44**, 181-198.

Perry, S.W., *et al.* (2011) Mitochondrial membrane potential probes and the proton gradient: a practical usage guide, *Biotechniques*, **50**, 98-115.

Pesole, G., *et al.* (2012) The neglected genome, *EMBO Rep*, **13**, 473-474.

Pessayre, D., *et al.* (2001) Mitochondria in steatohepatitis, *Semin Liver Dis*, **21**, 57-69.

Petros, J.A., *et al.* (2005) mtDNA mutations increase tumorigenicity in prostate cancer, *P Natl Acad Sci USA*, **102**, 719-724.

Pfannschmidt, T., Nilsson, A. and Allen, J.F. (1999) Photosynthetic control of chloroplast gene expression, *Nature*, **397**, 625-628.

Piko, L. and Taylor, K.D. (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos, *Dev Biol*, **123**, 364-374.

Plante, L. and King, W.A. (1994) Light and electron microscopic analysis of bovine embryos derived by in vitro and in vivo fertilization, *J Assist Reprod Genet*, **11**, 515-529.

Prihoda, J., *et al.* (2012) Chloroplast-mitochondria cross-talk in diatoms, *J Exp Bot*, **63**, 1543-1557.

Puthiyaveetil, S., Ibrahim, I.M. and Allen, J.F. (2013) Evolutionary rewiring: a modified prokaryotic gene regulatory pathway in chloroplasts, *Phil. Trans. R. Soc. B*, **This issue**.

Puthiyaveetil, S., *et al.* (2010) Transcriptional control of photosynthesis genes: the evolutionarily conserved regulatory mechanism in plastid genome function, *Genome biology and evolution*, **2**, 888-896.

Puthiyaveetil, S., *et al.* (2008) The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts, *Proc Natl Acad Sci U S A*, **105**, 10061-10066.

Race, H.L., Herrmann, R.G. and Martin, W. (1999) Why have organelles retained genomes?, *Trends Genet*, **15**, 364-370.

Radulovic, M., *et al.* (2010) CKS proteins protect mitochondrial genome integrity by interacting with mitochondrial single-stranded DNA-binding protein, *Mol Cell Proteomics*, **9**, 145-152.

Randerson, J.P. and Hurst, L.D. (2001) The uncertain evolution of the sexes, *Trends in Ecology & Evolution*, **16**, 571-579.

Rhee, H.W., *et al.* (2013) Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging, *Science*, **339**, 1328-1331.

Rich, P.R. (2003) The molecular machinery of Keilin's respiratory chain, *Biochemical Society transactions*, **31**, 1095-1105.

Rodriguez-Manas, L., *et al.* (2009) Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation, *Aging Cell*, **8**, 226-238.

Rozen, S. and Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In Krawetz, S. and Misener, S. (eds), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 365-386.

Sagan, L. (1967) On the origin of mitosing cells, *J Theor Biol*, **14**, 255-274.

Samuels, D.C., *et al.* (2010) Reassessing evidence for a postnatal mitochondrial genetic bottleneck, *Nature genetics*, **42**, 471-472; author reply 472-473.

Sang, J.H. (2001) *Drosophila melanogaster*: The Fruit Fly. In Reeve, E.C.R. and Black, I. (eds), *Encyclopedia of genetics*. Fitzroy Dearborn, London ; Chicago, Ill., pp. xix, 952 p.

Sarraf, S.A., *et al.* (2013) Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization, *Nature*, **496**, 372-376.

Sasaki, T., *et al.* (2008) Age-related increase of superoxide generation in the brains of mammals and birds, *Aging Cell*, **7**, 459-469.

Satoh, M. and Kuroiwa, T. (1991) Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell, *Exp Cell Res*, **196**, 137-140.

Schimper, A.F.W. (1883) Ueber die Entwickelung der Chlorophyllkörner und Farbkörper, *Bot. Zeit.*, **41**, 105-113, 121-131, 137-146, 153-162.

Schimper, A.F.W. (1885) Untersuchungen über die Chlorophyllkörner und die ihnen homologen Gebilde, *Jahrb. Wiss. Bot.*, **16**, 1-247.

Schmidt, O., Pfanner, N. and Meisinger, C. (2010) Mitochondrial protein import: from proteomics to functional mechanisms, *Nature reviews. Molecular cell biology*, **11**, 655-667.

Schrodinger, LLC (2010) The PyMOL Molecular Graphics System, Version 1.3r1.

Schwarzlander, M., *et al.* (2012) Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in Arabidopsis, *Plant Cell*, **24**, 1188-1201.

Searcy, D.G. (2003) Metabolic integration during the evolutionary origin of mitochondria, *Cell research*, **13**, 229-238.

Serviddio, G. and Sastre, J. (2010) Measurement of mitochondrial membrane potential and proton leak, *Methods Mol Biol*, **594**, 107-121.

Shao, Z.Y., *et al.* (2006) Mitochondrial genome of the moon jelly *Aurelia aurita* (Cnidaria, Scyphozoa): A linear DNA molecule encoding a putative DNA-dependent DNA polymerase, *Gene*, **381**, 92-101.

Shigenaga, M.K., Hagen, T.M. and Ames, B.N. (1994) Oxidative damage and mitochondrial decay in aging, *P Natl Acad Sci USA*, **91**, 10771-10778.

Shoubridge, E.A. (2002) The ABCs of mitochondrial transcription, *Nature Genetics*, **31**, 227-228.

Simpson, A. and Roger, A. (2004) The real 'kingdoms' of eukaryotes, *Current Biology*, **14**.

Smith, A.C., Blackshaw, J.A. and Robinson, A.J. (2012) MitoMiner: a data warehouse for mitochondrial proteomics data, *Nucleic Acids Res*, **40**, D1160-1167.

Sogin, M., *et al.* (1989) Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*, *Science*, **243**, 75-77.

Song, J.L. and Wessel, G.M. (2005) How to make an egg: transcriptional regulation in oocytes, *Differentiation*, **73**, 1-17.

Spikings, E.C., Alderson, J. and St John, J.C. (2007) Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development, *Biology of Reproduction*, **76**, 327-335.

St-Pierre, J., *et al.* (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain, *The Journal of biological chemistry*, **277**, 44784-44790.

Stewart, J.B., *et al.* (2008) Strong purifying selection in transmission of mammalian mitochondrial DNA, *PLoS biology*, **6**, e10.

Stock, D., Leslie, A.G. and Walker, J.E. (1999) Molecular architecture of the rotary motor in ATP synthase, *Science*, **286**, 1700-1705.

Stojkovic, M., *et al.* (2001) Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture, *Biol Reprod*, **64**, 904-909.

Sugimoto, T., *et al.* (2008) Caenorhabditis elegans par2.1/mtssb-1 is essential for mitochondrial DNA replication and its defect causes comprehensive transcriptional alterations including a hypoxia response, *Exp Cell Res*, **314**, 103-114.

Sugimoto, T., *et al.* (2008) Caenorhabditis elegans par2.1/mtssb-1 is essential for mitochondrial DNA replication and its defect causes comprehensive transcriptional alterations including a hypoxia response, *Exp Cell Res*, **314**, 103-114.

Taniguchi, K., Kajiyama, T. and Kambara, H. (2009) Quantitative analysis of gene expression in a single cell by qPCR, *Nat Methods*, **6**, 503-506.

Tarasenko, V.I., *et al.* (2009) Induction of Arabidopsis gdh2 gene expression during changes in redox state of the mitochondrial respiratory chain, *Biochemistry (Mosc)*, **74**, 47-53.

Taufer, M., *et al.* (2005) Is the Val16Ala manganese superoxide dismutase polymorphism associated with the aging process?, *J Gerontol A Biol Sci Med Sci*, **60**, 432-438.

Taylor, K.D. and Piko, L. (1995) Mitochondrial biogenesis in early mouse embryos: expression of the mRNAs for subunits IV, Vb, and VIIc of cytochrome c oxidase and subunit 9 (P1) of H(+)-ATP synthase, *Mol Reprod Dev*, **40**, 29-35.

Thompson, J.G., *et al.* (2000) Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro, *Journal of reproduction and fertility*, **118**, 47-55.

Thundathil, J., Filion, F. and Smith, L.C. (2005) Molecular control of mitochondrial function in preimplantation mouse embryos, *Mol Reprod Dev*, **71**, 405-413.

Timmis, J.N., *et al.* (2004) Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes, *Nat Rev Genet*, **5**, 123-135.

Tourte, M., Mignotte, F. and Mounolou, J.C. (1984) Heterogeneous distribution and replication activity of mitochondria in Xenopus laevis oocytes, *Eur J Cell Biol*, **34**, 171-178.

Tovar, J., *et al.* (2003) Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation, *Nature*, **426**, 172-176.

Treiber, N., *et al.* (2011) Accelerated aging phenotype in mice with conditional deficiency for mitochondrial superoxide dismutase in the connective tissue, *Aging Cell*, **10**, 239-254.

Trifunovic, A., *et al.* (2005) Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production, *P Natl Acad Sci USA*, **102**, 17993-17998.

Trifunovic, A., *et al.* (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase, *Nature*, **429**, 417-423.

Turner, G. and Muller, M. (1983) Failure to detect extranuclear DNA in Trichomonas vaginalis and Tritrichomonas foetus, *J Parasitol*, **69**, 234-236.

Van Blerkom, J. (2004) Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence, *Reproduction*, **128**, 269-280.

Van Blerkom, J. (2011) Mitochondrial function in the human oocyte and embryo and their role in developmental competence, *Mitochondrion*, **11**, 797-813.

Van Blerkom, J., *et al.* (2002) Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos, *Human Reproduction*, **17**, 393-406.

Van Blerkom, J., Davis, P.W. and Lee, J. (1995) ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer, *Hum Reprod*, **10**, 415-424.

Van Blerkom, J., Sinclair, J. and Davis, P. (1998) Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy, *Human Reproduction*, **13**, 2857-2868.

van der Giezen, M. (2009) Hydrogenosomes and mitosomes: conservation and evolution of functions, *J Eukaryot Microbiol*, **56**, 221-231.

van Hoek, A.H., *et al.* (2000) A mitochondrial ancestry of the hydrogenosomes of Nyctotherus ovalis, *Mol Biol Evol*, **17**, 202-206.

Van Raamsdonk, J.M. and Hekimi, S. (2009) Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans, *PLoS Genet*, **5**, e1000361.

Van Raamsdonk, J.M. and Hekimi, S. (2012) Superoxide dismutase is dispensable for normal animal lifespan, *P Natl Acad Sci USA*, **109**, 5785-5790.

Virbasius, J.V. and Scarpulla, R.C. (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis, *P Natl Acad Sci USA*, **91**, 1309-1313.

von Heijne, G. (1986) Why Mitochondria Need a Genome, *FEBS Lett.*, **198**, 1-4.

Wai, T., Teoli, D. and Shoubridge, E.A. (2008) The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes, *Nature genetics*, **40**, 1484-1488.

Wallace, D.C. (2010) Mitochondrial DNA mutations in disease and aging, *Environ Mol Mutagen*, **51**, 440-450.

Wallin, I.E. (1923) The mitochondria problem, *American Naturalist* **57**, 255-261.

Wang, Z., Gerstein, M. and Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics, *Nat Rev Genet*, **10**, 57-63.

Webb, R.J., *et al.* (2002) Gap-junctional communication in mouse cumulus-oocyte complexes: implications for the mechanism of meiotic maturation, *Reproduction*, **123**, 41-52.

Weber, J. and Senior, A.E. (2000) ATP synthase: what we know about ATP hydrolysis and what we do not know about ATP synthesis, *Biochim Biophys Acta*, **1458**, 300-309.

Weismann, A. (1889) *Essays Upon Heredity*. The Clarendon Press, Oxford.

Wilding, M., *et al.* (2001) Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos, *Human Reproduction*, **16**, 909-917.

Wilson, S.B., *et al.* (1996) Redox control of RNA synthesis in potato mitochondria, *Eur J Biochem*, **242**, 81-85.

Winkler, H.H. and Neuhaus, H.E. (1999) Non-mitochondrial ATP transport, *Trends Biochem Sci*, **24**, 64-68.

Wuichet, K., Cantwell, B.J. and Zhulin, I.B. (2010) Evolution and phyletic distribution of two-component signal transduction systems, *Current opinion in microbiology*, **13**, 219-225.

Yang, D., *et al.* (1985) Mitochondrial origins, *Proc Natl Acad Sci U S A*, **82**, 4443-4447.

Yang, T.W., Yang, Y.A. and Xiong, Z. (2000) Paternal inheritance of chloroplast DNA in interspecific hybrids in the genus Larrea (Zygophyllaceae), *Am J Bot*, **87**, 1452-1458.

Zhang, Y.Z., *et al.* (2008) Mitochondrial behavior during oogenesis in zebrafish: a confocal microscopy analysis, *Dev Growth Differ*, **50**, 189-201.

Zick, M., Rabl, R. and Reichert, A.S. (2009) Cristae formation-linking ultrastructure and function of mitochondria, *Biochim Biophys Acta*, **1793**, 5-19.

Zimprich, F., Ashworth, R. and Bolsover, S. (1998) Real-time measurements of calcium dynamics in neurons developing in situ within zebrafish embryos, *Pflugers Arch*, **436**, 489-493.

Zouros, E., *et al.* (1994) An unusual type of mitochondrial DNA inheritance in the blue mussel Mytilus, *P Natl Acad Sci USA*, **91**, 7463-7467.